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THE UNIVERSITY OF ALBERTA

THE TRANSPORT OF ANDROGENS
IN RAT PROSTATE

by

© PAUL S. RENNIE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "THE TRANSPORT OF ANDROGENS IN RAT PROSTATE", submitted by PAUL RENNIE in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate this Thesis to my friend, Richard Y. Sasaguchi (1942-1973) whose full scientific potential was never realized.

ABSTRACT

The principal objective of this study was to identify and partially purify the androgen-receptors of the ventral prostate gland of the rat. A second objective was to study the functional relationship between steroid-receptors and the transport of androgens across the nuclear membrane of prostatic cells.

The *in vitro* binding of [1,2-^{3}H]dihydrotestosterone and [1,2-^{3}H]testosterone to nuclear proteins was measured by gel filtration and association constants of the order of 10^7 M^{-1} were obtained. Castration caused a decrease in the binding of dihydrotestosterone to nuclear receptors and an increase in its binding to cytosol receptors.

Cytoplasmic and nuclear receptors were partially purified with chromatographic techniques employing cellulose phosphate and Sephadex G-200. Under *in vitro* conditions six types of steroid-receptors were found - four in cytosol and two in nuclei. However, two of the cytosol receptors were not observed *in vivo*. All forms of intracellular binding proteins were different from steroid receptors in serum.

In vivo binding studies demonstrated that only testosterone and dihydrotestosterone bind to steroid-receptors in cytosol and that only these two steroids are incorporated into the nucleus. The transport of steroids across the nuclear membrane was shown to depend on a concentrative process as most of the intracellular

radioactivity originating from a pulse injection of [1,2-^{3}H]testosterone was retained in nuclei. This property taken together with other considerations indicates the incorporation of androgens into nuclei is accomplished by an active transport system.

When the transfer of androgens from cytoplasm to nucleus was studied in vivo using pulse-chase methods, it was found that the cytosol and nuclear receptors were labelled in sequence and that the amounts of radioactive androgen lost from the former and gained by the latter were almost equal. These results are consistent with the concept that the combined action of cytosol and nuclear receptors is necessary for the transfer of steroid across the nuclear membrane. Further investigation of the various intracellular receptors revealed that cellulose phosphate Peak 2 receptors of cytosol and Sephadex Peak III receptors of nuclei are closely related in structure and appear to assume the role of steroid carriers in the cell. Therefore, it seems reasonable to conclude that the nuclear uptake of steroids is largely, if not entirely, dependent on the action of these particular molecules.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

cpm	counts per minute
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
x g	centrifugal force relative to gravity
Ka	association constant
Kp	partition coefficient
M	molar
mM	millimolar
μCi	microcurie
μg	microgram
μℓ	microliter
$NADP^+$, NADPH	nicotinamide adenine dinucleotide phosphate and its reduced form
nm	nanometers
pmoles	picomoles
RNA	ribonucleic acid
RNase	ribonuclease
S	Svedberg unit of sedimentation (10^{-13}cm/sec/dyne/g)
S.E.	standard error (of the mean)
Tris	tris (hydroxymethyl)aminomethane
Vo	void volume

CHAPTER I

INTRODUCTION

"Corpora non agunt nisi fixata"

(Paul Ehrlich, 1854-1915)

The binding of small molecules (ligands) such as vitamins and steroid hormones to molecular entities such as proteins is now understood to be an obligatory step in the expression of the biological activity of many essential compounds. Our insight into the importance of macromolecule-ligand interactions is attributable to the pioneer studies of Paul Ehrlich (Pilner, 1972). Results of his early research with dyes and proteins led him to postulate that low molecular weight substances are not biologically active unless they become attached to specific receptors (Corpora non agunt nisi fixata).

Heidenhain (1903) and Bechhold (1907) were the first to provide experimental evidence in support of this hypothesis. Heidenhain observed that serum albumin, when added to a solution of acidic azo dyes, interfered with the dialysis of dyes through parchment tubing. A reasonable interpretation of his results was that binding had occurred between the dye and the serum protein. Bechhold explored this phenomenon further, utilizing the technique of ultrafiltration. From his data he concluded that dyes, such as methylene blue, could be reversibly adsorbed to serum proteins. Since the inception of these studies the interaction of small molecules with specific binding proteins has proven to be relevant in the fields of enzymology, immunology, toxicology, oncology, and endocrinology.

1. Binding of Steroids to Serum Proteins: Early Studies

The first observation of reversible binding of steroids to proteins was reported by Oppenheimer (1913). While conducting *in vitro* experiments on the effects of digitalis on frog heart, he noted a decreased toxicity of the steroids when serum was included in the incubation medium. He surmised that his results were due to the formation of a steroid-protein complex. The adsorption of the steroids to serum had apparently reduced the effective amount of digitalis reaching the cardiac cells.

Unfortunately the significance of Oppenheimer's results was largely ignored until the early 1930's at which time Bennhold (1932) began a thorough investigation of protein-ligand interactions. As a result of his studies he was able to postulate that serum proteins act as vehicles for the transport of steroids and other small molecules to specific tissues. Upon delivery to target organs, the protein-ligand complex would dissociate and allow the transported moiety to exert its biological activity. This mechanism still remains as the accepted explanation for transport of steroids in the vascular system.

Following the discovery of sex hormones[1] in the late 1920's Brunelli (1934) conducted a series of dialysis experiments in which he demonstrated that estrogens could bind reversibly to serum proteins. However, it was not until fourteen years later that testosterone[2] was also shown to participate in similar protein binding interactions (Bischoff and Pilhorn, 1948; Barry et al., 1952). While this early work provided evidence for direct association between steroid hormones and serum proteins, there was a paucity of information concerning the fate of such hormones in their respective target tissues.

2. Binding of Androgenic Steroids to Intracellular Proteins: Survey of Recent Advances

The synthesis of radioactive steroid hormones provided the opportunity for investigators to examine the localization of such compounds in peripheral tissues. In the case of androgens, Barry et al. (1952) found a significant amount of radioactivity present in the seminal vesicles of the rat after a single injection of radioactive testosterone. Similar studies by Greer (1959) confirmed Barry's results and demonstrated that the ventral prostate of the rat was also capable of accumulating labelled androgen. Furthermore he found that the radioactive testosterone disappeared rapidly from the blood and was retained to a much higher degree by the accessory sex glands than by adrenals or muscle. Apparently a mechanism existed for the selective uptake of androgens by target organs.

The retention and subcellular distribution of radioactive androgens in various tissues of the rat was first examined by Harding and Samuels (1962). These authors reported that although the total radioisotope concentration in the ventral prostate of the rat was not much greater than that of the blood, the relative amount of unconjugated steroid in this organ was nearly twice that measured in the blood. Thus in the ventral prostate there was preferential incorporation from the circulation of neutral androgens rather than the more polar, conjugated forms.

Harding and Samuels next examined the subcellular distribution of radioactive androgens under conditions in which EDTA (ethylene-diaminotetracetic acid) was either included in or deleted from the homogenizing medium. In the presence of EDTA, most of the labelled androgen appeared in the microsomal fraction; less than 5% of the radioactivity was recovered in the nuclei. This observation was paralleled by an EDTA stimulated shift of RNA from the nuclear to the microsomal fraction. In the absence of EDTA, most of the labelled androgen was detected in the high speed cytoplasmic supernatant fraction and in the nuclear fraction.

The intracellular localization of androgens in the uropygial (preen) gland of the duck was studied by Wilson and Loeb (1965). The preen gland is an accessory sex organ which possesses the necessary criteria of a target tissue in that it selectively incorporates circulating androgens. With this biological system Wilson and Loeb were able to show that nuclei had a greater avidity for radioactive androgens than did the ribosomes. Furthermore, closer examination of the nuclear fraction revealed that most of the radioactivity was associated with an euchromatin fraction which contained approximately 90% of the radioactive label but less than 10% of the nuclear DNA. Using cesium chloride density gradient centrifugation techniques, they were able to separate the DNA from a protein fraction which contained most of the radioactivity. From their work they inferred that hormone responsive tissues contain intranuclear proteins which bind androgens reversibly.

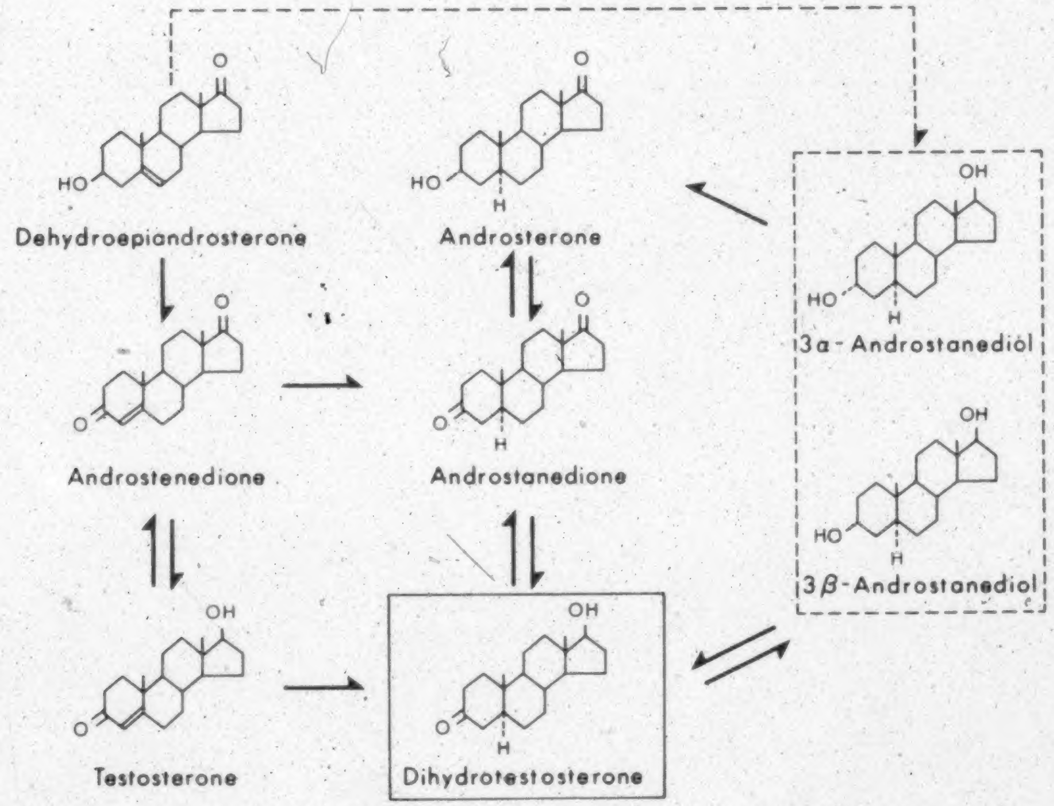
The occurrence of such nuclear androgen receptors in the rat ventral prostate was reported by Bruchovsky and Wilson (1968b). After injection of radioactive testosterone into normal or functionally hepatectomized rats a significant amount of the radioisotope recovered in the ventral prostate was associated with the nuclear fraction. Using high molar salt extraction of nuclei, followed by gel filtration of nuclear extracts, they were able to demonstrate the presence of a macromolecular fraction which was eluted from the gels coincident with a peak of radioactive hormone. This radioactive complex was salt extractable from the nuclei at neutral or basic pH and was sensitive to pronase. Much of this work contradicted the studies by Mangan et al. (1968) who had proposed that prostatic DNA itself served as the source of binding sites for androgens.

Examination of the steroid metabolites recovered in these experiments revealed that dihydrotestosterone and to a lesser extent, testosterone, were the only androgens bound to nuclear receptor protein in any significant amounts. In an earlier publication Bruchovsky and Wilson (1968a) had reported that after an injection of radioactive testosterone most of the radioactivity recovered from rat prostate was in the form of androstanediol, androsterone, and dihydrotestosterone. The latter androgen was detected only in the prostate, seminal vesicles, preputial gland, kidney, and to a small extent, in the plasma. Since dihydrotestosterone was the predominant radioactive steroid recovered in prostate, and since it was also the major form of androgen bound to nuclear receptors, the possibility was raised that dihydrotestosterone is an active form of testosterone. It was suggested that the mechanism of action of testosterone involves, at least two reactions, namely the conversion of testosterone to dihydrotestosterone, and the binding of dihydrotestosterone to an intracellular protein receptor.

3. Formation of Dihydrotestosterone in Androgen Responsive Tissues

The finding that relatively large amounts of dihydrotestosterone are formed in the prostate has served as the impetus for a great deal of research concerning testosterone metabolism in peripheral tissues. Several groups have confirmed that extensive formation of dihydrotestosterone occurs in other secondary sexual

ANDROGEN METABOLISM IN THE RAT VENTRAL PROSTATE



tissues (Tveter and Aakvaag, 1969; Shimazaki *et al*., 1969; Wilson and Walker, 1969; Wilson and Glogna, 1971; Bardin *et al*., 1970). In many bioassay systems dihydrotestosterone has been shown to have a greater potency than testosterone with respect to DNA synthesis (Bruchovsky, 1972; Lesser and Bruchovsky, 1973; Schmidt *et al*., 1972) and RNA synthesis (Davies *et al*., 1972). It seemed highly pertinent therefore, that a number of other potent natural androgens were found to give rise to dihydrotestosterone in prostate. On the basis of this evidence it was suggested that all androgenic compounds may exert their action through the common formation of dihydrotestosterone (Bruchovsky, 1971). However, there are reasons to doubt that dihydrotestosterone serves as the ultimate factor in determining androgen action. For example, Baulieu *et al*. (1968), found that testosterone and dihydrotestosterone elicit different cytological effects on explants of prostate maintained *in vitro*. However, their studies did indicate that dihydrotestosterone specifically stimulated proliferation of target cells (Baulieu, 1970). It is not clear, therefore, whether testosterone functions as a circulating pre-hormone (i.e. it is converted to the active form, dihydrotestosterone, in peripheral target organs), or whether it plays its own defined role within target cells. The same question may be raised concerning the action of other androgens.

4. Binding of Dihydrotestosterone in Rat Prostate

Shortly after the discovery of intranuclear androgen receptors in the ventral prostate, Unhjem *et al*. (1969) reported that prostatic cytoplasm also contains proteins which bind steroid hormones. Cytoplasmic extracts, incubated with radioactive dihydrotestosterone, were spun through sucrose density gradients and the migration of radioactive peaks was measured. Two peaks of radioactivity were detected, one with a sedimentation coefficient of 9.3S, and a second with a coefficient of 4.5S. Confirmation of dihydrotestosterone binding proteins in prostatic cytoplasm was provided by others (Stern and Eisenfeld, 1969; Mainwaring, 1969).

Fang *et al*. (1969) conducted a series of comprehensive *in vivo* and *in vitro* studies to characterize both the cytoplasmic and the nuclear binding proteins of rat prostate. Using sucrose density gradient centrifugation they were able to show that the dihydrotestosterone binds to the protein of nuclear extracts obtained after *in vivo* incubation, and that this protein migrates with a sedimentation coefficient of 3S. They also reported that the cytoplasmic high speed supernatant contains a dihydrotestosterone binding protein which has a sedimentation coefficient of 3.5S. The binding proteins were found to be temperature sensitive, to have a pH optimum around 7.4, to possess a high degree of specificity for dihydrotestosterone, and to be sensitive to proteases but not to nucleases.

When incubations were done with minced prostate, the radioactive steroid protein complexes isolated from the cytosol (105,000 x g supernatant) or nuclear fractions had the same respective sedimentation coefficients as those observed in *in vivo* labelled extracts. Similar results were obtained when cytosol protein and nuclei were incubated together with radioactive steroid. However, if isolated prostatic nuclei were incubated in buffer with radioactive steroid, binding of steroid to nuclear protein was not observed. Upon replacement of the buffer with prostatic cytosol as the suspending medium, one could then detect a 3S nuclear steroid-protein complex. The latter result was also achieved if the 3.5S steroid-protein complex isolated from prostatic cytoplasm was incubated with unlabelled nuclei. They concluded that a cytoplasmic entity, presumably the androgen binding protein was necessary for the uptake and retention of dihydrotestosterone by nuclei. Furthermore they postulated that nuclear uptake of androgens was a result of a two step process whereby the hormones first are bound to a cytoplasmic receptor and then are transported, as part of a biomolecular complex, into the nucleus.

Although the concept of a two step mechanism for the incorporation of androgens into nuclei has become the working hypothesis of many investigators, significant discrepancies exist between the results obtained by Fang *et al*. (1969) and those reported by others. Several authors (Mainwaring, 1969; Baulieu and Jung, 1970; Jung and Baulieu, 1971) have shown that in addition to the 3.5S steroid-protein complex in the cytosol, an 8S species is also present. Moreover, two independent laboratories (Unhjem, 1970; Jung and Baulieu, 1971) have demonstrated that specific binding of dihydrotestosterone to prostatic nuclear proteins occurs *in vitro* even in the absence of added cytoplasmic factors. However, they did find that the endogenous levels of androgenic binding proteins in isolated nuclei declined as a function of the duration of castration.

5. Binding of Androgens in Pathological Tissues

Although the importance of the intracellular binding of steroids remains obscure, it is tentatively accepted that binding reactions play an integral role in regulating the growth of target tissues. It is reasonable to expect, therefore, that disorders of growth involving hormone responsive tissues may be associated with an alteration of the characteristic binding activity observed in normal tissue.

Benign prostatic hypertrophy is an age dependent endocrine disorder in which there is a significant enlargement of the prostate (Ofner, 1968). Siiteri and Wilson (1970) found that the accumulation of dihydrotestosterone was almost five times greater in the prostates of patients with benign prostatic hyperplasia than in the prostates of normal controls. Both types of prostatic tissue however, had similar capacity to form dihydrotestosterone from testosterone. This finding suggested that increased uptake and retention rather than increased metabolism of testosterone is responsible for the hyperplastic change. Other studies (Giorgi *et al.*, 1971, 1972) support this conclusion and implicate the intracellular binding of dihydrotestosterone as the main factor causing the increased retention of steroid in benign prostatic hypertrophy.

There is considerable evidence that hormone dependent tumours (i.e. tumours which regress after castration) possess specific sex steroid binding proteins whereas hormone independent tumours of secondary sexual tissue show reduced or altered steroid binding activity (McGuire and Julian, 1971; McGuire *et al.*, 1972; Shyamala, 1972). Bruchovsky and Meakin (1973) have shown that in the Shionogi SC-115 mouse mammary adenocarcinoma, there is a positive correlation between androgen dependency and cytoplasmic binding of androgens. Autonomous tumour lines exhibit both diminished binding and reduced nuclear accumulation of the steroid when compared to dependent lines.

Another less prevalent endocrine disorder, the testicular feminization syndrome (Weisberg *et al.*, 1970; Rosenfield *et al.*, 1971) is also associated with abnormal levels of androgen binding (Gehring and Tomkins, 1971; Bullock *et al.*, 1971; Bullock and Bardin, 1970; Wilson and Goldstein, 1972).

6. Functional Significance of Intracellular Steroid Binding Proteins:, Hypotheses

Since intracellular proteins which bind steroids appear to be essential for maintenance of hormonal control of male reproductive organs, the question arises as to the function of these macromolecules. There are at least three possible roles that binding entities may fulfill (Williams-Ashman and Reddi, 1971).

First, these proteins may be concerned with activation of sex hormones. Steroid hormones are small simple molecules which have a relatively limited chemical information content. In order to account for their diverse array of biochemical responses it would be necessary for them to be associated with a molecule of higher information content - such as a protein. Hence the true active form of the hormone could well be the specific cytoplasmic protein-androgen complex.

Secondly, the binding proteins may function as carriers for the transport of specific androgens into the nucleus. Either the whole cytoplasmic androgen-protein complex enters the nucleus or the androgen component of the complex is transferred at the nuclear membrane level to another androgen binding protein that is restricted to the nucleus. While it is possible to imagine variations on this idea, the end result would be the delivery of a particular androgen into the nucleus. The steroids themselves could then elicit a series of biochemical events within the nucleus involving DNA synthesis, RNA synthesis or alterations in the permeability of the nuclear membrane.

A third alternative is that the androgens facilitate the nuclear uptake of the cytoplasmic binding proteins (i.e. the converse of the second hypothesis). When the hormone binds to a specific protein in the cytoplasm, the protein undergoes an alteration which facilitates its entry into the nucleus. Upon entering the target cell nucleus the protein itself, rather than the steroid, initiates the appropriate cellular response. Thus the steroid may operate in a permissive manner to ensure intracellular transport of specific proteins.

The three possibilities outlined above are not mutually exclusive. It is conceivable that both the unbound and the protein bound steroid may influence different biochemical processes within the nucleus. Also, the nucleus may not be the only site at which androgens, either unbound or bound to proteins, influence cellular activity. A still further possibility does exist in which the intracellular binding proteins assume a negative role. These proteins may function to regulate the amount unbound or free steroid present within the cell. In this storage capacity the androphilic proteins would allow only a small amount of active free steroid to realize its biocatalytic function.

7. Rationale and Objectives

From the material presented in this Chapter it is evident that one approach to the study of the early sites and mechanisms of action of androgens is based on the concept that androgens are bound only in characteristic target organs and that binding is related to subsequent physiological actions of the hormone.

Accordingly the main objective of this work was to purify and characterize the protein receptors for androgens in rat prostate. A second principal objective was to examine the presumed function of these receptors in mediating the action of testosterone and dihydrotestosterone.

Footnotes

[1] The earliest preparation and therapeutic usage of androgens and estrogens dates back to the medieval Chinese alchemists. Modern sex hormone endocrinology has its roots in the work of Ascheim and Zondek (Williams-Ashman and Reddi, 1971).

[2] The trivial names used are testosterone, Δ^{4}-androsten-17β-ol-3-one; dihydrotestosterone, 5α-androstan-17β-ol-3-one; androstanediol, 5α-androstan-3α, 17β-diol; estradiol, 1,3,5 (10)-estratrien-3, 17β-diol; epitestosterone, Δ^{4}-androsten-17β-ol-3-one; androsterone, 5α-androstan-3α-ol-17-one; androstenedione, Δ^{4}-androstene-3,17-dione; androstanedione, 5α-androstane-3,17-dione; cyproterone acetate, 1,2α-methylene-6-chloro-$\Delta^{4,6}$-pregnadien, 3,20-dione, 17β-acetate; estrone, 1,3,5 (10)-estratrien-3-ol-17-one; estriol, 1,3,5 (10)-estratrien-3,16α, 17β-triol.

CHAPTER II

MATERIALS AND METHODS

A. Introduction

This chapter is a comprehensive survey of methods for the isolation of subcellular fractions and the recovery of associated intracellular receptor molecules. In each of the following chapters a "Materials and Methods" section is included which summarizes the procedures used in connection with the work that is described.

B. Materials

1. Buffers and Related Solutions

The principal buffers used in the isolation and incubation of tissue fractions are listed in Table 2.1. The titration of the Tris-EDTA buffer (Richardson, 1966) to pH 7.0 with 12 N HCl was done at room temperature with a Radiometer (Copenhagen, Denmark) pH meter. All solutions were prepared in one liter or greater quantities and stored in a cold room at 4° C.

2. Chemicals

General laboratory chemicals and reagents were purchased from Fisher Scientific Co. (Montreal, Quebec). Ultra-pure sucrose was obtained from Schwarz/Mann (Orangeburg, New York). Non-radioactive steroids were purchased from Steraloids, Inc. (Pawling, New York)

TABLE 2.1

Solution		
a) Tris EDTA buffer, pH 7.0	.01 M Tris (hydroxymethyl) aminomethane (THAM) 5 x 10^{-5} M Ethylenediamine tetraacetic acid (EDTA) 5 x 10^{-3} M $MgCl_2 \cdot 6H_2O$ 5 x 10^{-4} M 2-Mercaptoethanol	*in glass distilled water
b) .05 M NaCl	.05 M NaCl	in Tris EDTA buffer, pH 7.0
.15 M NaCl	.15 M NaCl	
.60 M NaCl	.60 M NaCl	
.80 M NaCl	.80 M NaCl	
1.15 M NaCl	1.15 M NaCl	
c) .88 M sucrose	.88 M sucrose and 1.5 mM $CaCl_2 \cdot 2H_2O$	in Tris EDTA buffer, pH 7.0
1.8 M sucrose	1.8 M " " 0.5 mM " "	
2.2 M sucrose	2.2 M " " " " "	
.25 M sucrose	.25 M " " 1.5 mM " "	

except cyproterone acetate which was a gift from the Schering Corporation (Bloomfield, New Jersey). Bovine serum albumin and calf thymus DNA were obtained from Sigma (St. Louis, Missouri).

3. Balances

Three types of balances were used to determine the weight of materials. The Mettler P1200N balance was used for samples that weighed more than 500 mg. Samples weighing between 5 and 500 mg were measured with the Mettler H10Tw balance. And finally, the Mettler Micro Gram balance was used to determine the weight of very small amounts of materials (< 5 mg).

C. Preparation of Tissue Extracts

1. Experimental Animals

Male rats of the Wistar strain were purchased from Woodlyn Laboratories, (Guelph, Ontario). The rats were housed in the Animal Center of the Clinical Sciences Building where they were maintained on a diet of Rockland Rat Chow and water *ad libitum*.

Animals weighing 250 to 300 g were routinely used. Castration was performed through a scrotal incision while the rats were under ether anesthesia. The castrated rats used in *in vivo* experiments were eviscerated and functionally hepatectomized (Hotta and Chaikoff, 1955) immediately prior to the intravenous administration of radioactive steroid. At the appropriate time, the animals were killed by decapitation.

2. Homogenization of Tissue

The procedures employed for the homogenization of prostatic tissue, and subsequent isolation of nuclei, were based on the methods described by Bruchovsky and Wilson (1968a). Immediately after the experimental animals were sacrificed, their ventral prostates were dissected free of enveloping capsular tissue and placed in a beaker on ice. All further preparative procedures were carried out at 0 to 4° C.

The prostates were weighed, and then chopped with a TC-2 Sorvall tissue slicer (Allied Scientific Co., Scarborough, Ontario). Examination of the tissue under the light microscope (American Optical Corp., Buffalo, New York) after this process revealed large clumps of intact cells and small pieces of fibrous tissue. In order to remove contaminating serum and prostatic secretion from the preparation, the tissue mince was suspended in 5 ml of 0.25 M sucrose solution and then pelleted at 800 x g for 15 min in a Sorvall RC2-B Superspeed refrigerated centrifuge (SS-34 rotor, r_{avg}, 5.4 cm). The washed pulp was resuspended in 7 to 10 ml of 0.25 sucrose and transferred to a Dounce homogenizer (Kontes Glass Co., Vineland, New Jersey). The cells were ruptured by 25 strokes with a loosely fitting plunger and filtered through two layers of gauze. After a further 15 strokes with the tightly fitting glass plunger, the crude homogenate was centrifuged at 800 x g for 15 min in the Sorvall centrifuge. The resultant supernatant constituted the "crude cytoplasmic fraction", and 800 x g pellet was designated the "crude nuclear fraction".

3. Preparation of Cytosol Protein

Cytosol was prepared from the 800 x g supernatant through further centrifugation at 10,000 x g for 20 min in a Sorvall centrifuge and then at 105,000 x g for 120 min (SW41 rotor, r_{avg}, 10.78 cm) in a Beckman-Spinco L2-65B preparative ultracentrifuge. The final supernatant was processed according to the requirements of the particular experiment. In many instances, the cytosol fraction was brought to 80% saturation with ammonium sulphate (v/v) by stepwise addition of ammonium sulphate crystals over a period of 1 hr with the temperature controlled at 4° C. Precipitated protein was collected by centrifugation at 17,000 x g for 20 min in a Sorvall centrifuge and dissolved in 1 ml of Tris-EDTA buffer, pH 7.0.

4. Isolation of Prostatic Nuclei

Purification of prostatic nuclei was accomplished by centrifugation through a discontinuous sucrose gradient. The crude nuclear pellet was first suspended in 0.88 M sucrose solution (25 ml per tube) and then gently layered over a discontinuous sucrose gradient consisting of 5 ml of 1.8 M sucrose and 5 ml of 2.2 M sucrose. The tubes were centrifuged at 53,000 x g for 90 min (SW27 rotor, r_{avg}, 11.56 cm) in a Beckman-Spinco ultracentrifuge. On completion of centrifugation, the nuclear pellet was suspended in

Tris-EDTA buffer, containing 50 mM NaCl. Aliquots were taken for counting of nuclei and, where applicable, for measurement of radioactivity. The nuclei were stained with a few drops of methylene blue (1% w/v) and then counted at least twice under the light microscope using a haemocytometer (American Optical Corp., Buffalo, New York). Nuclei were considered sufficiently pure for further experimentation if fewer than 5% of them had visible cytoplasmic tags. In later experiments it was found that the presence of less than 1% androstandiol in this fraction could also be used as a criterion of nuclear purity. Nuclear preparations that did not meet these requirements were either discarded or were respun through sucrose gradients.

5. Preparation of Nuclear Extracts

The isolation of a chromatin fraction from purified nuclei was accomplished by sonication and extraction with a salt solution of high molarity. The prostatic nuclei were first suspended in an hypotonic salt solution (Tris-EDTA buffer, pH 7.0, with 0.05 M NaCl) and then allowed to swell for 10 min. Following this period, the nuclei were ruptured with a Bronwill Biosonik III (Bronwill Scientific, Rochester, New York) sonicator at a setting of 50 with 5/16" probe (yellow code). Four 5 second pulses were adequate to disrupt the nuclei. An equal volume of 1.15 M NaCl (in Tris-EDTA buffer, pH 7.0) solution was added to the sonicated nuclear fraction, giving a final NaCl concentration of 0.6 M. After

30 min in the cold, the solution was sedimented in a Sorvall centrifuge at 17,000 x g for 20 min. The supernatant was withdrawn with a Pasteur pipet and the pellet was re-extracted with a small amount of 0.6 M NaCl solution. When radioactive nuclear samples were used, the extraction procedure was repeated until at least 90% of the radioisotope was present in the supernatant; in general, this was achieved with two extractions. The supernatants obtained from repeated salt extractions were pooled. The final sample thus obtained is referred to as the "nuclear extract".

D. Column Chromatography

1. Preparation of Sephadex Gels

Sephadex G-200 and coarse Sephadex G-25 (Pharmacia Fine Chemicals, Montreal, Quebec) were prepared according to the instructions provided by the manufacturer. The Sephadex powder was added slowly to a large beaker of distilled water and was occasionally stirred with a glass rod. After all the dry material had been immersed in water, the beaker was covered with parafilm and the slurry was allowed to swell for 3 to 5 days at room temperature. Fines were removed from the swollen gel immediately prior to use.

2. Preparation of Sephadex G-25 Columns

Glass columns for Sephadex G-25 chromatography were manufactured by the University of Alberta Technical Services. Before the columns were packed with gel, they were silanized by immersion for one hour in a solution containing dimethyldichlorosilane in toluene (10% v/v). Following this, they were rinsed with toluene and washed with methanol. This procedure successfully prevented the adhesion of extraneous material to the column walls.

The packing of Sephadex G-25 into columns was performed at ambient temperature in order to prevent the formation of air bubbles. A small piece of silanized glass wool was first placed in the tapered end of the column which was closed off with a small section of polyethylene tubing. The column was next partially filled with water and a small amount of slurry. As the gel particles settled under gravity, water was removed from the upper portion of the column and more gel was added. This cycle was repeated until the gel matrix had attained a specific bed volume height. The packed columns were then removed to the cold room where they were equilibrated under gravity with an appropriate buffer solution.

3. Gel Filtration with Sephadex G-25

After the columns had been equilibrated with 300 to 400 ml of buffer, suitable cytoplasmic or nuclear extracts were applied. Samples (0.5 to 1.5 ml) were gently layered onto the gel and allowed to enter the Sephadex under gravity. The surface of the gel was then washed with 2 to 3 ml of buffer to ensure passage of all the sample into the gel bed. Following this, the column was connected to a buffer reservoir and the flow rate was adjusted to 1.8 to 2 ml per min. The eluted fractions were collected either manually or with a Brinkmann

Linear II fraction collector (Brinkman Instruments, Rexdale, Ont.). The fraction of interest was the void volume (V_o) which contained the protein-androgen complexes. Detection of the position of this fraction was accomplished by one of the following methods. (i) The location of the radioactive peak associated with the void volume could be found by measuring the cpm of small aliquots from each fraction. (ii) The 280 mμ absorbing material in the V_o could be monitored with an Isco Model UA2 ultraviolet analyzer (Allied Scientific Co., Scarborough, Ont.). (iii) The progress of the macromolecule fraction through the column could be detected visually when Blue Dextran 2000 (Pharmacia) was mixed with the sample just prior to chromatography. After the appropriate fractions had been collected, they were assayed for protein and radioactivity.

4. Gel Filtration with Sephadex G-200

The method for packing a Sephadex G-200 column was identical to that described for Sephadex G-25. However, in the case of the former, the gel bed (1 cm x 90 cm) filled the entire column (Glenco Precision Bore, O.H. Johns, Toronto, Ontario). The Sephadex G-200 column was equilibrated with buffer in an upward direction at 4° C using an LKB Type 4912A peristaltic pump (LKB, Uppsala, Sweden).

The apparatus used for applying a sample to the column consisted of a 5 cc disposable syringe (Standard Hospital Supply Ltd., Edmonton, Alberta), an 18 gauge needle, and a section of polyethylene tubing which was connected to one aperture of a 3 way stopcock at the lower end of the column. The sample, usually less than 1.5 ml, was inserted into the syringe and was allowed to enter the polyethylene tubing under gravity. 3 ml of the appropriate buffer was then placed in the syringe to ensure sufficient pressure to force the sample along the tubing and eventually into the bottom of the column. When the flow of fluid through the sample applicator apparatus had stopped, the stopcock was turned to permit entry of buffer from a reservoir. The peristaltic pump was switched on and the elution of proteins was done in an upward direction with a flow rate of 8 to 10 ml per hr. After a fixed number of tubes were collected, the fraction collector (Ultro Rac LKB 7000) activated automatic shut down of the system. Aliquots from the collected fractions were taken for the measurement of radioactivity and protein.

5. Preparation of Cellulose Phosphate

Whatman P11 cellulose phosphate (W & R Balson, England) was prepared by first stirring 100 g of dry fiber with 1000 ml of 0.5 N NaOH for 30 min at room temperature. The cellulose phosphate was then transferred to a Buchner funnel where the NaOH was removed by gentle suction. Repeated washings with distilled water were used to reduce the pH of the suspension to 8, and then the cellulose phosphate was resuspended in 1000 ml of 0.5 N HCl. The mixture was stirred for 30 min and washed as before with distilled water until the pH was 4. After this step, the acidic cellulose phosphate was suspended in 500 ml of Tris-EDTA buffer, pH 7.0, stirred for 15 min and then titrated to pH 7.0 with 6 N KOH over a period of 6 hours at room temperature. Columns (Pharmacia), 1 cm x 15 cm, prepared from this material were equilibrated at 4° C with 300 ml of Tris-EDTA buffer, pH 7.0.

6. Cellulose Phosphate Chromatography

Samples, from which excess salt had been removed by passage through Sephadex G-25 columns were layered on top of the ion exchange material and then allowed to permeate the column bed under gravity. Following sample application, the columns were washed with 20 to 25 ml of Tris-EDTA buffer, pH 7.0. Proteins were then eluted at a flow rate of 0.4 ml per min with a linear ionic gradient consisting of 0 to 0.8 M NaCl in Tris-EDTA buffer, pH 7.0. The protein and radioactive content of each 4.2 ml fraction collected were determined.

E. Identification of Steroids from Tissue Extracts

1. The Extraction of Steroids from Aqueous Solutions

The extraction of steroids from aqueous solutions was accomplished by an adaptation (Bruchovsky and Wilson, 1968a; Bruchovsky, 1972) of the method described by Folch *et al*. (1957). Aliquots from tissue fractions were agitated manually in five volumes of chloroform-methanol (2:1, v/v) and then centrifuged at 1000 x g for 10 min in a MSE Multex Centrifuge (O.H. Johns Scientific, Toronto) at room temperature. The upper aqueous phase was removed and the lower phase was shaken with an equal volume of upper phase solvent (chloroform-methanol-water, 3:48:47 by vol.). Following centrifugation, the upper phase was again removed and the extraction was repeated one more time. The resultant lower phase was passed through a teflon Millipore filter (Millipore Ltd., Montreal, Quebec) with a 5 micron pore size. The filtrate was taken to dryness under nitrogen and resuspended in a small volume of chloroform. Portions of the sample were taken for analysis by thin layer chromatography.

2. Thin Layer Chromatography

The identification of steroids was accomplished by thin layer chromatography using an aqueous suspension of either silica gel H (Merck, Brinkmann, Instruments, Toronto) (30 g 172 ml) or aluminum oxide, neutral, Type T (Brinkman) (30 g 142 ml). Glass plates (20 cm x 20 cm) were coated with a 250 mμ layer of the material, and activated at 100° C in Fisher Isotemp oven (Fisher Scientific) for 1 hour.

Separation of androstandiol, testosterone, androsterone, dihydrotestosterone, androstanedione, and androstenedione was achieved by two upward developments of alumina oxide plates in cyclohexane-ethyl acetate - acetic acid (60:40:0.1 by vol.). Good resolution of 17β-estradiol, estriol and estrone was possible on silica gel plates after one development in benzene-ethanol (8:2 v/v). Appropriate unlabelled standards (25 μg of each compound) were added to the radioactive tissue extracts to ensure detection of each spot.

Following chromatography, the plates were dried and sprayed with a 0.01% solution of morin in methanol (w/v) and then examined under ultraviolet light. The location of each standard was noted and the plate was divided into horizontal bands which were then scraped into counting vials. One ml of methanol together with 10 ml of 0.4% diphenyloxazole in toluene (w/v) were added to the vials and the samples were measured for radioactivity. The validity of the above technique for determining androgen and estrogen metabolites of the rat prostate has been demonstrated using gas-liquid chromatography and recrystallization techniques (Bruchovsky, 1971; 1972).

F. Measurement of Protein and DNA

1. Protein Determinations

Protein was measured by a color reaction (Lowry et al., 1951) and by differential ultraviolet absorption (Layne, 1957). Samples assayed by the Lowry method were diluted to a final volume of 1 ml with distilled water. Suitable blanks and protein standards (5 to 50 µg of bovine serum albumin) were prepared which had an identical concentration of buffer and other small molecules to that present in the unknown samples. Each reaction vessel received 5 ml of a solution containing 0.01% $CuSO_4 \cdot 5\ H_2O$, 0.02% sodium tartrate, and 2% Na_2CO_3 in 0.1 N NaOH, and was allowed to stand for 10 min at room temperature. Next, 0.5 ml of 1 N Folin reagent were added to the samples, which were then placed in a 37° C water bath for 30 min. At the end of this incubation, the absorption of each sample at 500 nm was determined using a Bausch and Lomb Spectronic 20 (Rochester, New York). By plotting absorption against the protein concentration of the standards, it was possible to interpolate the amount of protein in the unknown samples.

Although the Lowry method provided relatively accurate quantitation of protein concentrations, it was, however, time consuming and required the construction of a standard curve for each set of data. Because of this inconvenience a somewhat similar technique was adopted that permitted the protein assay of several samples in a short time. This procedure involved reading the optical density of samples at 260 and 280 nm against appropriate blanks on the Zeiss PMQ spectrophotometer (Carl Zeiss Canada Ltd., Don Mills, Ontario). The values at each wavelength were recorded and applied to a formula described by Layne (1957) (see Appendix). This method for determining protein concentration was used only with fractions eluted from cellulose phosphate and Sephadex G-200.

2. DNA Determinations

The preparation of DNA containing samples was done according to standard procedures (Maggio *et al.*, 1963). DNA was measured by the diphenylamine method (Schneider, 1957) with calf thymus DNA as a reference standard. After development of the colour reaction, the optical density of the samples was determined at 600 nm with a Spectronic 20. A standard curve was constructed and from this, an estimate of the amount of DNA present in each of the biological extracts was obtained.

G. Measurement of Radioactivity

1. Radioactive Materials

[1,2-^{3}H]testosterone (5 mCi/.032 mg), [1,2-^{3}H]-dihydrotestosterone (1 mCi/6.0 μg), and [6,7-^{3}H]17β-estradiol (5 mCi/0.029 mg) were purchased from New England Nuclear (Boston, Mass.). Purity was checked by thin layer, gas liquid and liquid chromatography. The steroids were considered acceptable only when the purity exceeded 90%. Solutions for intravenous injections were prepared as follows: to each 150 μCi of radioactive steroid in ethanol-benzene solution was added 15 μℓ of 5% polyoxyethylene sorbitan monopalmitate, and the solution was dried under nitrogen. The dry preparation was then dissolved in a suitable amount of distilled water containing 5% ethanol. Each rat was injected with 250 μℓ of such a solution.

2. Liquid Scintillation Counting

Liquid scintillation counting was carried out with either a diphenyloxazole-toluene solution (4 g of diphenyloxazole per liter of toluene) for non aqueous samples or a Bio-solv-cocktail (6 g of diphenyloxazole; 1 liter of toluene; 75 ml water and 116 g of BBS-3 (Beckman Instruments) for aqueous samples. Samples were deposited in glass vials (Value Vials, Beckman Instruments), and the radioactivity was measured in a Beckman LS-250 automatic liquid scintillation system. External standardization was used to correct for quench when necessary. Otherwise the efficiency of tritium detection was usually about 50% in the diphenyloxazole-toluene solution and 35% in the Bio-Solv solution.

CHAPTER III

IN VITRO BINDING STUDIES

A. Introduction

The interaction of male sex hormones with cellular receptors has been the subject of intense study (Williams-Ashman and Reddi, 1971) but surprisingly little information is available on the comparative binding parameters of the principal androgens, testosterone and dihydrotestosterone. While it is generally assumed that the cytosol receptor sites for testosterone and dihydrotestosterone are the same, it remains possible that the observed variation in binding activity of the two hormones (Fang *et al*., 1969) results from the presence of distinct receptor sites for each hormone. Since such knowledge is of potential importance in the interpretation of binding data, experiments were undertaken to obtain detailed measurements of the reaction of these hormones with receptors in prostatic cytoplasm and nuclei *in vitro*.

B. Materials and Methods

1. Conditions for Routine *In Vitro* Incubations

Cytosol and nuclear fractions were isolated from prostatic tissue as described in Chapter II. For the measurement of hormone binding to nuclear receptors, approximately 1 to 2 x 10^7

nuclei were suspended in 1 ml of Tris-EDTA buffer (pH 7.0) with 50 mM NaCl, containing either [1,2-^{3}H]testosterone or [1,2-^{3}H]dihydrotestosterone. To facilitate solution of high concentrations of androgens in aqueous media, a maximum of 2% ethanol (v/v) was mixed with the buffer in some experiments. No adverse effects on binding were noted in control experiments with this quantity of ethanol. *In vitro* incubations were routinely carried out at 25° C for 90 min in a Dubnoff shaking water bath (Precision Scientific, Chicago, Ill.).

Cytosol samples (105,000 x g cytoplasmic supernatant) were also incubated with radioactive steroids at 25° C for 90 min. Approximately 500 μg of cytosol protein in 0.25 M sucrose solution was mixed with Tris-EDTA buffer, pH 7.0, containing ^{3}H-androgen, and the final volume was adjusted to 1 ml with buffer.

2. Measurement of Steroid Binding

Following the incubation period nuclei were sonicated and extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The nuclear extract was then applied to a Sephadex G-25 column (1 cm x 40 cm) and eluted at a flow rate of 1.8 to 2.0 ml per min with either 0.6 M NaCl in Tris-EDTA buffer, pH 7.0, or buffer with no added salt. Cytosol samples were chromatographed under identical conditions. In each case, the radioactivity and protein in the void volume fraction of the columns were measured.

3. *In Vitro* Incubations with Enzymes

Deoxyribonuclease I (bovine pancreatic) (Mann), ribonuclease Type III-A (bovine pancreatic) (Mann) and pronase, B grade, (Sigma) were added to incubation mixtures to test the sensitivity of the binding complexes. The conditions are described in the text.

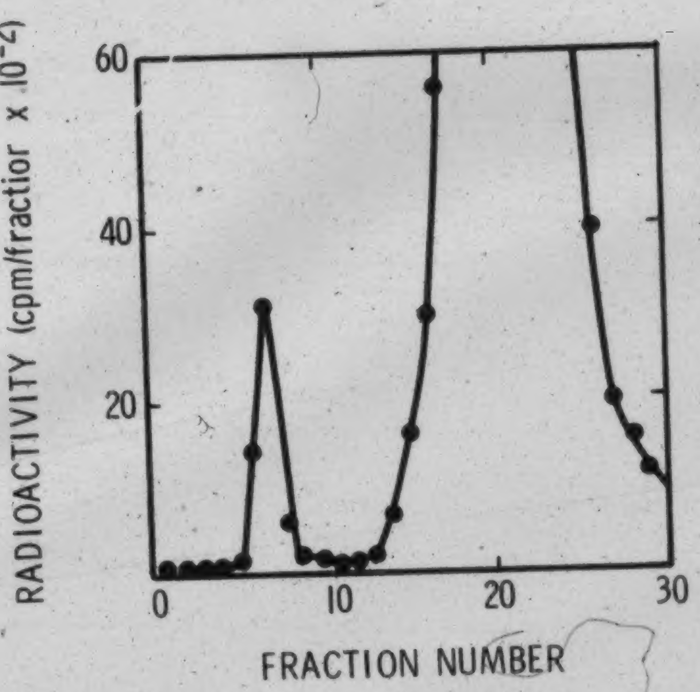
C. Results

1. Establishment of Conditions for Steroid Binding *In Vitro*

Many of the techniques available for the measurement of the binding of radioactive ligand to macromolecules are either time consuming (e.g. density-gradient centrifugation and equilibrium dialysis) or lack sufficient accuracy (e.g. charcoal adsorption) for precise quantitative studies. Gel filtration procedures, however, provide a relatively rapid and yet accurate evaluation of such interactions (Wood and Cooper, 1970). Sephadex G-25 columns have been used to measure steroid binding in rat prostate (Bruchovsky and Wilson, 1968b), and recently gel filtration with Sephadex G-25 has been shown to be a superior method for the quantitation of steroid hormone interactions with proteins (Jungblut *et al*., 1972).

When prostatic protein extracts are incubated with ^{3}H-androgen and then run on a Sephadex G-25 column one observes a profile of radioactivity as shown in Fig. 3.1. The first peak of radioactivity (the void volume fraction) contains the receptor

Fig. 3.1. Separation on Sephadex G-25 of [1,2-^{3}H]dihydrotestosterone into bound and free fractions. Approximately 500 μg of cytosol protein were incubated with 2.5 x 10^{-8} M [1,2-^{3}H] dihydrotestosterone for 90 min at 25° C and then applied to a 1 cm x 40 cm Sephadex column. Fractions of 2 ml were eluted with Tris-EDTA buffer, pH 7.0, and the radioactivity in each was measured.


RADIOACTIVITY (cpm/fraction x 10^{-2})
60
40
20
0
10
20
30
FRACTION NUMBER

proteins, whereas the second peak corresponds to the elution volume observed when free ^{3}H-androgen is passed through the column. Accordingly, it was possible to measure the amount of radioactive steroid bound to proteins by sampling the void volume fraction obtained after gel filtration of cellular extracts.

During the initial phases of this work, the effects of incubation temperature and elution buffer were studied. Nuclei and cytosol proteins were incubated with radioactive testosterone or dihydrotestosterone for 90 min at 4, 25, and 37° C (Table 3.1). Following incubations nuclear and cytosol extracts were prepared and then chromatographed on columns of Sephadex G-25 using the appropriate elution buffer. Although binding of androgen could be demonstrated at 4° C, maximal binding was achieved at higher temperatures. No appreciable difference was noted in the binding at 25° or 37°, but because of the instability of *in vivo* binding at 37° C (Bruchovsky and Wilson, 1968b), the lower temperature was considered more suitable for *in vitro* studies. The presence of 0.6 M NaCl in the elution buffer used during gel-filtration had no discernible effect upon the amount of radioisotope bound to receptors. Maximum binding was achieved during incubation periods of 60 to 90 min.

2. Effects of Column Length

After the determination of appropriate incubation conditions, experiments were conducted in order to estimate the amount of "on-column" dissociation that occurs when steroid-receptor

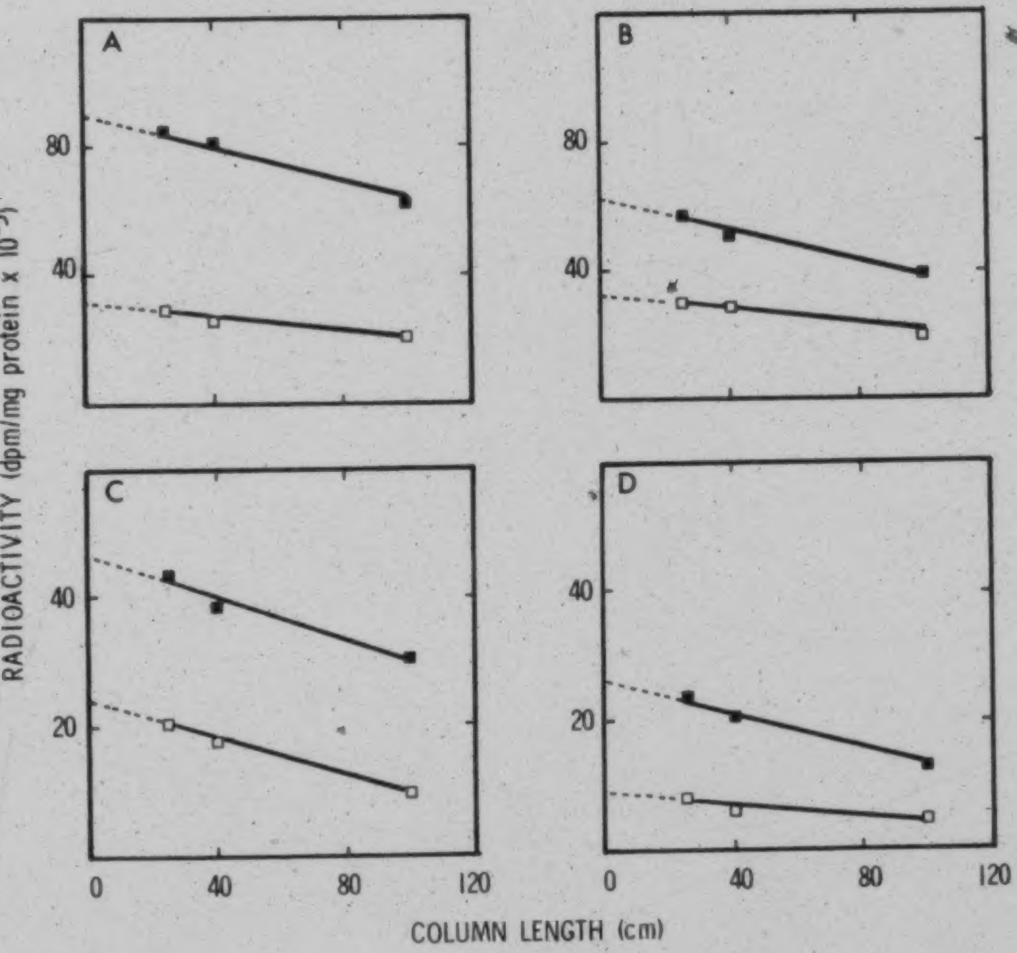
TABLE 3.1

THE EFFECTS OF INCUBATION TEMPERATURE AND ELUTION BUFFER ON INTRACELLULAR BINDING OF ANDROGENS

[NaCl] (M)	Temp. (°C)	Testosterone CYTO	Testosterone NUC	Dihydrotestosterone CYTO	Dihydrotestosterone NUC
		(dpm/mg Protein x 10^{-4})			
0.0	4	2.4	1.4	3.9	3.3
	25	3.0	1.5	5.8	4.6
	37	3.2	1.5	5.8	5.0
0.6	4	2.2	1.3	3.6	3.2
	25	2.8	1.6	5.5	4.7
	37	2.9	1.5	5.9	4.9

Nuclei and cytosol proteins were incubated with 4×10^{-8} M of either [1,2-^{3}H]dihydrotestosterone or [1,2-^{3}H]testosterone at 4°, 25° or 37° C for 90 min. Duplicate samples of the appropriate extracts were run on Sephadex G-25 columns in which the eluant consisted of Tris-EDTA buffer, pH 7.0, or Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The radioactivity and protein content in the void volume fraction of each column was determined. Each value represents the mean of two or more experiments.

Fig. 3.2. The effects of column size on the recovery of [1,2-^{3}H]testosterone and [1,2-^{3}H]dihydrotestosterone bound to cytosol and nuclear proteins. The appropriate subcellular fractions from intact rats were incubated at 25° C for 90 min with [1,2-^{3}H]-dihydrotestosterone or [1,2-^{3}H]testosterone and then analyzed for protein binding on Sephadex G-25 columns of lengths 25, 40, and 100 cm. In cytosol experiments the columns were eluted with Tris-EDTA buffer, pH 7.0; nuclear extracts were eluted with the Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The concentration of radioactive androgen was either 5 x 10^{-8} M (■) or 2 x 10^{-8} M (□). Each point represents the mean of two experiments. Radioactivity recovered: Panels A and B, dihydrotestosterone bound to cytosol and nuclear extracts respectively; panels C and D, testosterone bound to cytosol and nuclear extracts respectively.

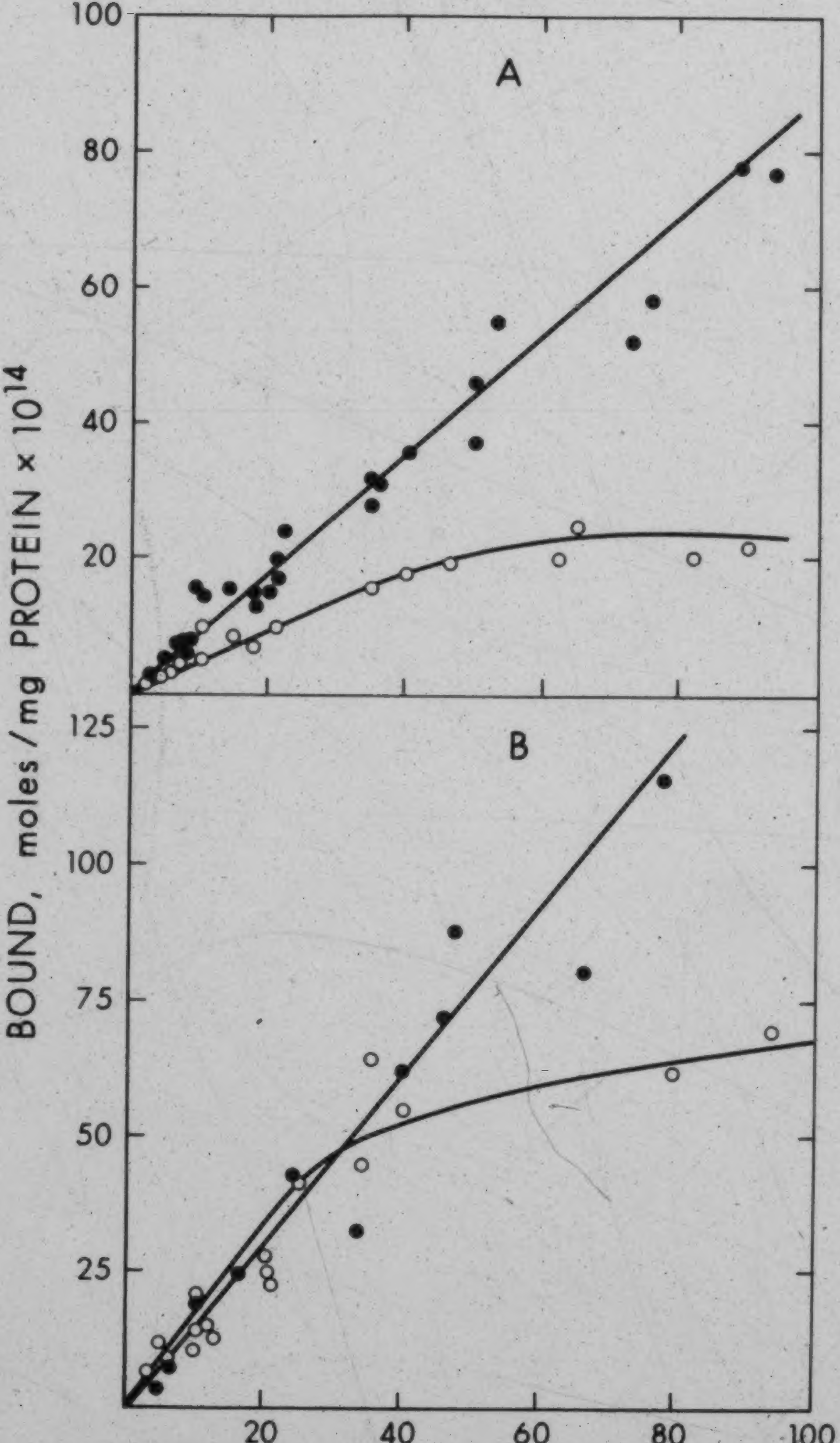

A
B
C
D
80
40
80
40
40
20
40
20
0
40
80
120
0
40
80
120
RADIOACTIVITY (dpm/mg protein x 10^{-3})
COLUMN LENGTH (cm)

complexes are passed through Sephadex columns. Nuclei and cytosol protein were incubated at 25° for 90 min with [1,2-^{3}H]testosterone or [1,2-^{3}H]dihydrotestosterone at concentrations of 2 x 10^{-8} M and 5 x 10^{-8} M. The labelled extracts were then applied to Sephadex G-25 columns of dimensions 1 cm x 25 cm, 1 cm x 40 cm, and 1 cm x 100 cm, and subsequently analyzed for steroid binding. The results shown in Fig. 3.2 indicate that the amount of ^{3}H-steroid recovered in the void volume fraction varies inversely as a function of column height. Since this relationship is linear, it was possible to extrapolate the recovery to zero column height and hence obtain a more accurate estimate of the binding reaction at equilibrium. The calculation of a correction factor for on-column dissociation was done relative to the 1 cm x 40 cm Sephadex column, as this size was most frequently used in gel filtration experiments. The binding of dihydrotestosterone to cytosol and nuclear receptors was underestimated with these columns by approximately 14 and 18 per cent respectively. Similarly, the levels of testosterone recovered in the void volume fraction of 1 cm x 40 cm columns after nuclear or cytosol experiments was 19 to 22 per cent less than that estimated for zero column height. The application of these correction factors to the raw binding data is only permissible if the rate of dissociation during gel filtration is constant at all steroid concentrations. Since extrapolation to zero column height at two different hormone concentrations indicated similar rates of on-column dissociation, such an assumption appears valid, as any differences are probably less than the inherent experimental error. An adaptation of this procedure has recently been described by Godefroi and Brooks (1973), and their results confirm the validity of correcting for "on-column" dissociation in the manner described in this section.

3. Determination of Association Constants

Experiments were next performed to determine the affinity constants for the binding of androgens to receptors. Cytosol preparations from normal rats were incubated with varying concentrations of radioactive testosterone and dihydrotestosterone for 90 min at 25° C, and then the amount of radioactive hormone bound to protein was determined by gel filtration on Sephadex G-25. Purified nuclei were also incubated with varying concentrations of tritiated testosterone and dihydrotestosterone for 90 min at 25° C. Following incubation, the nuclei were washed in Tris-EDTA buffer, pH 7.0, and extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The amount of protein-bound hormone in the nuclear extract was measured via gel filtration. As can be seen from the results shown in Fig. 3.3 it was not possible to saturate the cytosol receptor sites for either [1,2-^{3}H]testosterone or [1,2-^{3}H]-dihydrotestosterone in the concentration range up to 10^{-7} M. Even when the concentration was increased to 7×10^{-7} M the linear relationship was maintained. Conversely, the nuclear binding of [1,2-^{3}H]testosterone and [1,2-^{3}H]dihydrotestosterone reached an

Fig. 3.3. Binding of [1,2-^{3}H]testosterone and [1,2-^{3}H]-dihydrotestosterone *in vitro*. Prostatic tissue from 5 to 10 normal rats was homogenized in 0.25 M sucrose with a ball-type Dounce homogenizer. The crude homogenate was then separated into nuclear and cytosol fractions by the centrifugation steps described in Chapter II. Approximately 1.0 x 10^7 nuclei or 500 µg cytosol protein were incubated at 25° C for 90 min in one ml of Tris-EDTA buffer, pH 7.0 containing various concentrations of radioactive steroid as shown. Following the incubation period, nuclei were sonicated and extracted with Tris-EDTA buffer, pH 7.0 containing 0.6 M NaCl. The amount of bound hormone was determined by gel-filtration on Sephadex G-25. Panel A, binding of [1,2-^{3}H]-testosterone; panel B, binding of [1,2-^{3}H]-dihydrotestosterone. ●———● cytosol; ○———○ nuclei.


A
B
BOUND, moles / mg PROTEIN × 10^{14}
STEROID CONCENTRATION × 10^9 M
100
80
60
40
20
125
100
75
50
25
20
40
60
80
100

apparent plateau at approximately 5 x 10^{-8} M. Thus the binding reactions for both hormones exhibited qualitative similarities.

Scatchard plots (Scatchard, 1949) of the nuclear binding data, shown in Fig. 3.4, yielded estimates of the association constant (Ka) for testosterone and dihydrotestosterone. The values obtained were 1.1 x 10^7 M^{-1} and 3.5 x 10^7 M^{-1}, respectively, which suggested that the binding of each compound to chromatin protein was highly specific. Moreover, it was calculated that there were 1700 specific binding sites per nucleus for testosterone and 3400 for dihydrotestosterone in keeping with the molecular ratios observed *in vivo* (Bruchovsky and Wilson, 1968b; Fang *et al*., 1969). Although Fang and Liao (1971) have reported that nuclear binding occurs only in the presence of cytosol factors, Unhjem (1970) has shown that cytosol factors are not critical. The results obtained in this investigation (i.e. Figs. 3.3 and 3.4) indicate that significant binding takes place when nuclei are incubated in the absence of cytosol and thus confirm the latter observation.

4. The Binding of Testosterone and Dihydrotestosterone as a Function of Receptor Site Concentration

Since the above results demonstrate that the amount of hormone bound to cytosol protein increases in a linear fashion as a function of steroid concentration, it was of interest to see whether the gel-filtration binding assay could also detect changes in the

Fig. 3.4. Determination of association constants for the binding of [1,2-^{3}H]testosterone and [1,2-^{3}H]-dihydrotestosterone to nuclear receptors. Experiments were performed as described in the legend to Fig. 3.3. Concentrations of bound and free hormone were determined on Sephadex G-25 gel and the data were plotted using the relationship bound/free = K (bound/mg protein) + Kn, where K = slope and the number of binding sites/mg protein = n. Panel A, [1,2-^{3}H]testosterone; panel B, [1,2-^{3}H]-dihydrotestosterone.

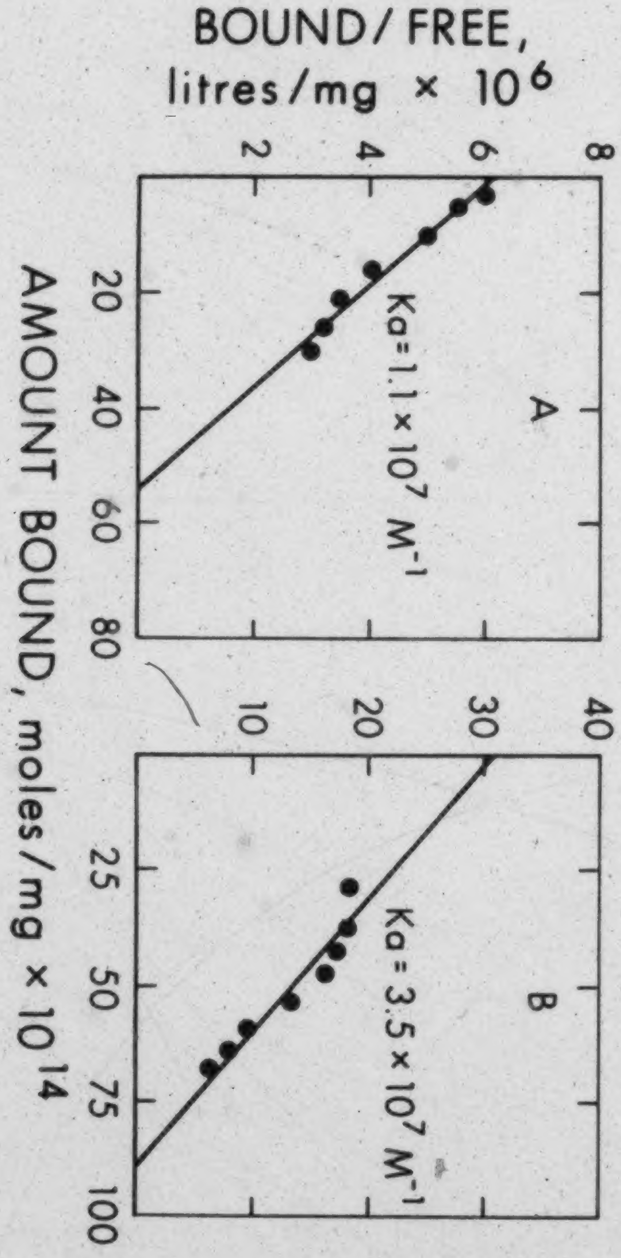
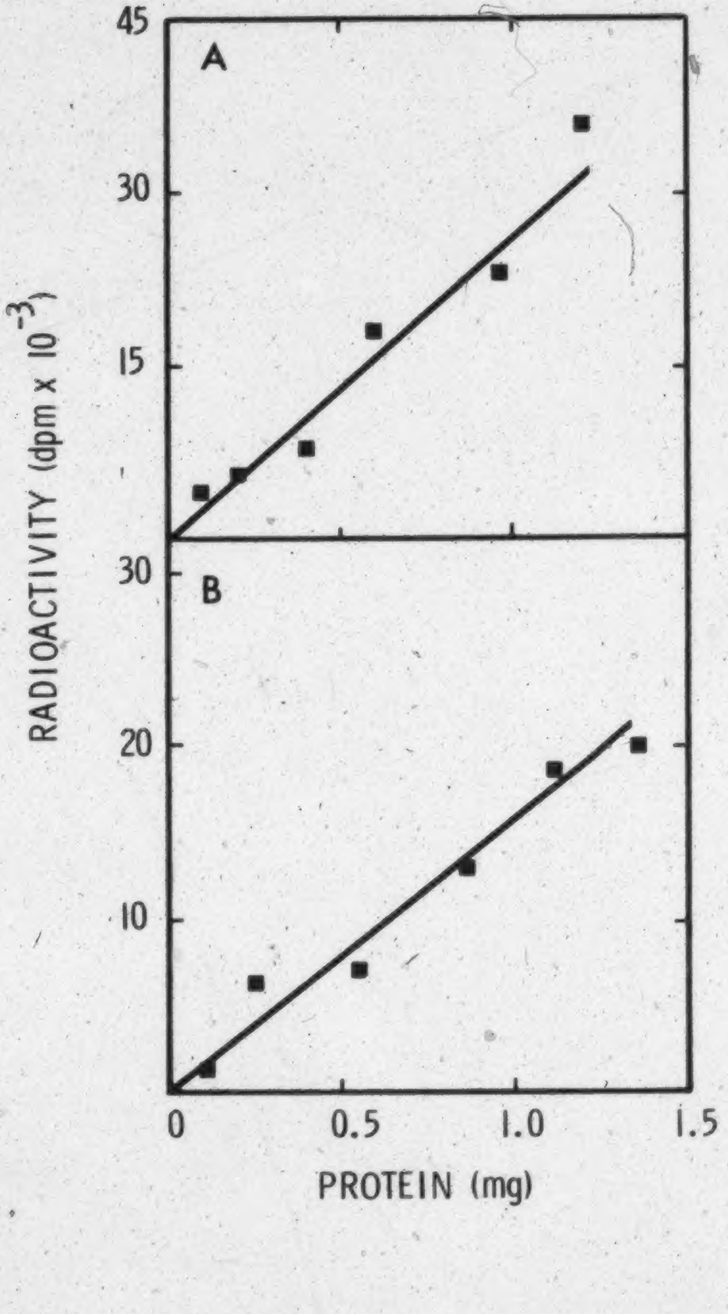

BOUND/ FREE,
litres/mg × 10^6
8
6
4
2
20
40
60
80
A
Ka=1.1 × 10^7 M^{-1}
40
30
20
10
25
50
75
100
B
Ka = 3.5 × 10^7 M^{-1}
AMOUNT BOUND, moles/mg × 10^{14}

Fig. 3.5. Binding of [1,2-^{3}H]dihydrotestosterone and [1,2-^{3}H]-testosterone to cytosol receptors as a function of protein concentration. Cytosol extracts containing different amounts of protein were incubated with ^{3}H-steroid at a concentration of 2×10^{-8} M and then measured for binding as before. Panel A, dihydrotestosterone bound to cytosol protein; panel B, testosterone bound to cytosol protein.


A
B
45
30
15
30
20
10
0
0.5
1.0
1.5
RADIOACTIVITY (dpm x 10^{-3})
PROTEIN (mg)

concentration of cytoplasmic receptor sites. Therefore, experiments were done in which varying amounts of cytosol protein from rat prostate were incubated at 25° C for 90 min with a fixed concentration (2×10^{-8} M) of either [1,2-^{3}H]testosterone or [1,2-^{3}H]-dihydrotestosterone. The results shown in Fig. 3.5 indicate that the amount of radioactive steroid bound to receptors increase linearly with the amount of protein present in the incubation mixture. Hence, Sephadex G-25 chromatography is a relatively sensitive technique for detecting fluctuations in both receptor site concentration and steroid concentration.

5. Identification of Steroids Bound to Receptors after *In Vitro* Incubations

Several investigators have shown that in the rat prostate testosterone and dihydrotestosterone are metabolized to other androgenic steroids under both *in vitro* and *in vivo* conditions (Bruchovsky and Wilson, 1968a; Bruchovsky, 1971; Moore and Wilson, 1972). However, when *in vitro* incubations of steroids with prostatic tissue are performed, the presence of NADP$^+$ and a NADPH$_2$-generating system are necessary for the reduction of testosterone to dihydrotestosterone (Baulieu *et al*., 1968) and for the conversion of dihydrotestosterone to androstandiol (Unhjem, 1970). Although these cofactors were not added to the incubation mixtures used in this study, the possibility existed that endogenous levels of NADPH$_2$ were sufficient to effect some metabolism of testosterone

TABLE 3.2

IDENTIFICATION OF STEROIDS AFTER *IN VITRO* INCUBATIONS

Fraction	Sample	^{3}H-Steroids Incubated	
		Testosterone	Dihydrotestosterone
		% Recovered as T	% Recovered as DHT
I Cytosol	Total	91	89
	Bound	93	87
II Nuclei	Total	95	87
	Bound	92	90

Following standard *in vitro* incubations with radioactive androgens, aliquots from the unfractionated cytosol and nuclear extracts and their respective androgen protein complexes were taken for steroid extractions. Steroid identification was performed using thin-layer chromatography as described in Chapter II. Results are expressed as per cent of the original ^{3}H-steroid recovered after *in vitro* incubations.

and dihydrotestosterone. Therefore experiments were conducted in which the steroids bound to receptors and those present in the unbound form were identified. Following *in vitro* incubations with $[1,2\text{-}^{3}H]$testosterone and $[1,2\text{-}^{3}H]$dihydrotestosterone aliquots were taken for extractions of steroids; the remaining samples were subjected to gel-filtration, after which the void volume fractions were also extracted. Identification of the steroids recovered in these experiments was accomplished by thin-layer chromatography (Chapter II). The results are shown in Table 3.2. Between 87 and 95 per cent of the radioactive androgens used in the binding experiments were recovered in their original unmetabolized form. Clearly, no significant metabolism of the steroid substrates occurred during these *in vitro* incubations.

6. Susceptibility of Steroid Binding to Pronase

In order to verify that nuclear and cytoplasmic receptors were composed largely of protein material (Bruchovsky and Wilson, 1968b; Fang *et al.*, 1969) the sensitivity of the hormone binding reaction to several enzymes was tested. Samples of cytosol or nuclear extracts were incubated at 25° C for 30 min with the appropriate radioactive hormone. Following this interval, they were incubated a further 60 min at 25° C without added enzyme, or with 100 μg of ribonuclease, deoxyribonuclease, or pronase. On completion of incubation, the levels of ^{3}H-steroid bound to receptors was measured using gel filtration. From the results shown

TABLE 3.3

RECOVERY OF BOUND RADIOACTIVITY FOLLOWING INCUBATION OF STEROID-RECEPTOR COMPLEXES WITH DIGESTIVE ENZYMES

Enzyme	Cytosol		Nuclei	
	T	DHT	T	DHT
	Radioactivity Recovered (%)			
None	100	100	100	100
Pronase	20	15	25	30
DNase	92	110	104	89
RNase	95	93	98	105

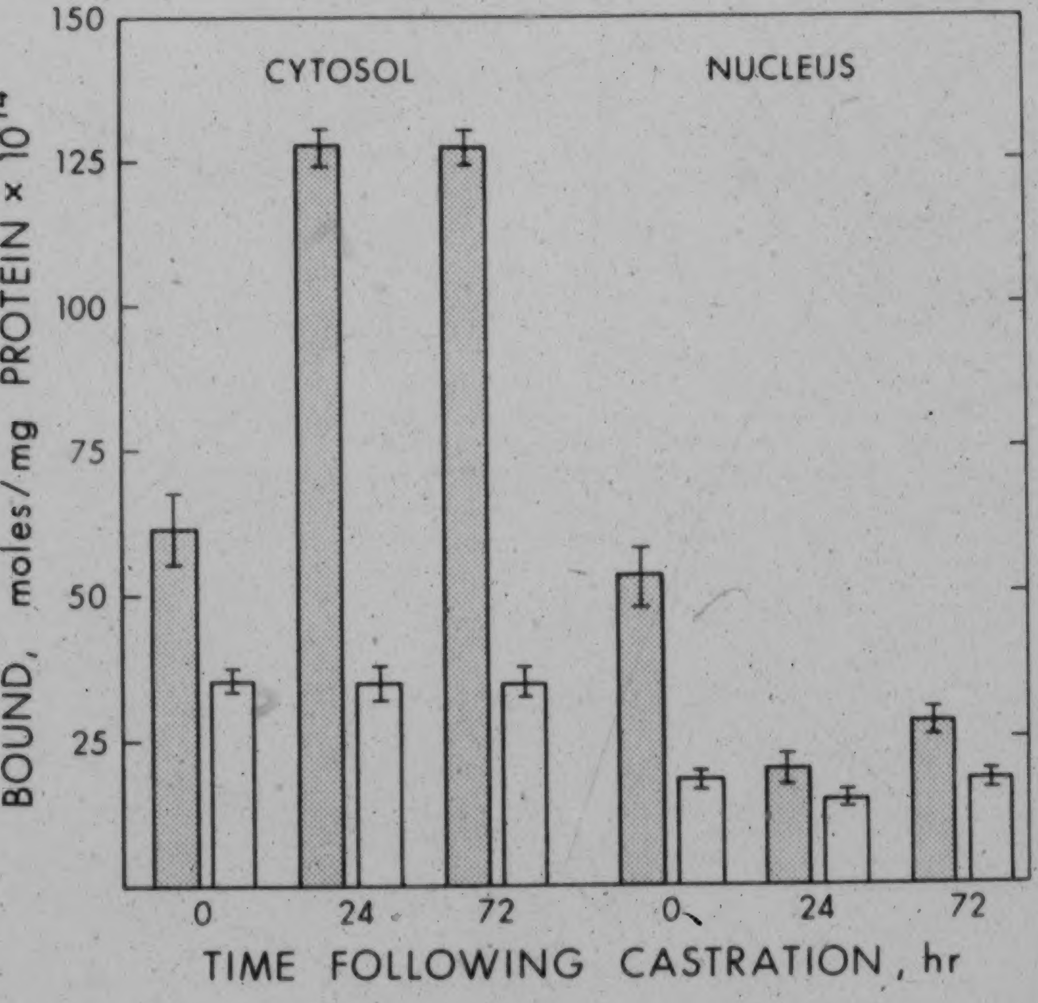
Samples containing either 200 μg of nuclear protein or 500 μg of cytosol protein were incubated with 4 x 10^{-8} M [1,2-^{3}H]testosterone or [1,2-^{3}H]dihydrotestosterone for 30 min at 25° C. At this time 100 μg of pronase, deoxyribonuclease, or ribonuclease were added, and the samples were incubated a further 60 min at 25°. Binding was measured in the usual manner using gel filtration. In the cytosol experiments the control values (100%) for binding were 2.8 x 10^4 dpm and 1.6 x 10^4 dpm for dihydrotestosterone and testosterone respectively. Similarly control values of 9.4 x 10^3 dpm of dihydrotestosterone and 2.8 x 10^3 dpm of testosterone were obtained in nuclear binding experiments. Each figure represents the mean of two experiments.

in Table 3.3 it is evident that only pronase treatment caused a pronounced reduction in the radioactivity recovered in the void volume fractions. This confirms that the receptor molecules are proteins.

7. Effects of Castration on Binding of Testosterone and Dihydrotestosterone

While the *in vitro* binding reactions for testosterone and dihydrotestosterone are qualitatively similar (Fig. 3.3) it was unknown whether the receptors for each are identical or different. In order to resolve this problem, the effects of castration on cytosol and nuclear binding were compared. It was expected that if dihydrotestosterone and testosterone were bound to the same sites on a single receptor protein, any shift in the binding of dihydrotestosterone caused by castration would be accompanied by a parallel shift in the binding of testosterone. Constant amounts of cytosol and purified nuclei obtained from castrated rats were incubated with hormone substrates added to a concentration of 4.0×10^{-8} M, which is close to the concentration of testosterone in the blood and prostate of some species (Gloyna *et al*., 1970; Siiteri and Wilson, 1970; Rivarola and Migeon, 1966) and within the concentration range where high affinity binding is expected (Fig. 3.3). From the results shown in Fig. 3.6 it can be seen that each time interval studied, the binding of [1,2-^{3}H]-dihydrotestosterone always exceeded the binding of [1,2-^{3}H]testosterone.

Fig. 3.6. Effects of castration on the binding of [1,2-^{3}H]-testosterone and [1,2-^{3}H]dihydrotestosterone. Nuclear and cytosol preparations were obtained from male rats castrated at 0, 24, and 72 hours prior to the beginning of the experiment. Approximately 1.0 x 10^7 nuclei or 500 μg cytosol protein were incubated at 25° C for 90 min in one ml of Tris-EDTA buffer, pH 7.0 containing 4.0 x 10^{-8} M radioactive testosterone or dihydrotestosterone. Nuclei were extracted with Tris-EDTA buffer, pH 7.0 containing 0.6 M NaCl. The amount of bound hormone in the various fractions was determined by gel-filtration on Sephadex G-25. Open bars, amount of [1,2-^{3}H]-testosterone bound, closed bars, amount of [1,2-^{3}H]-dihydrotestosterone bound. The standard error of the mean value from several experiments is shown for each time interval.



BOUND, moles/mg PROTEIN × 10^{14}
150
125
100
75
50
25
CYTOSOL
NUCLEUS
0
24
72
0
24
72
TIME FOLLOWING CASTRATION, hr

Furthermore the binding of [1,2-^{3}H]dihydrotestosterone was increased in cytosol and decreased in nuclei at 24 and 72 hours following castration. No corresponding changes were observed in the binding of [1,2-^{3}H]testosterone which remained relatively constant throughout the experiment. The specific change in the binding of dihydrotestosterone in response to castration suggests that the receptors for testosterone are not the same as those for dihydrotestosterone or alternatively, that the experimental conditions required to demonstrate the _in vitro_ binding of each hormone are different.

D. Discussion

In this chapter it was demonstrated that both testosterone and dihydrotestosterone bind to proteins in cell free extracts of rat prostate. Binding experiments with both hormones yielded association constants which indicate high affinity binding to nuclear protein (Fig. 3.3 and 3.4). The values are lower than values suggested by other authors (Fang _et al_., 1969; Ritzen _et al_., 1971), but they compare favourably with the association constants obtained for the binding of 17β-estradiol to endometrium (Zimmering _et al_., 1970). Since the estimate of 3400 receptor sites per nucleus for dihydrotestosterone is close to the approximations of 2000 (Fang _et al_., 1969) and 6000 (Mainwaring and Peterken, 1971) reported by others, the K_A value of 3.5×10^7 M^{-1} is probably significant.

The failure to saturate cytoplasmic receptor with either testosterone or dihydrotestosterone may be due to the presence of non-specific binding sites. Proteins possessing sites of high capacity yet low affinity for steroids have been postulated by Georgi _et_ _al._ (1972). Baulieu and Jung (1970) isolated a binding protein from rat prostate which was both non-specific and non-saturable at hormone concentrations comparable to physiological levels.

The presence of distinct receptor sites for testosterone and dihydrotestosterone was suggested by some of the data. From the Scatchard plots (Fig. 3.4) it appears that the number of binding sites (from the intercept on the abcissa) in nuclei differs for each steroid. Furthermore, the finding that castration produces a shift in the receptors for dihydrotestosterone but not for testosterone (Fig. 3.6) is consistent with the concept of separate sites. On the other hand, the results may also be interpreted to suggest that this _in_ _vitro_ system falls short of providing optimal conditions for the binding of testosterone.

CHAPTER IV

THE CHARACTERIZATION OF ANDROGEN-RECEPTOR COMPLEXES FORMED IN VITRO

A. Introduction

Within minutes of the administration of testosterone to castrated rats, dihydrotestosterone is recovered from prostatic cytosol in association with a 3 to 4S protein (Fang et al., 1969; Mainwaring, 1969; Ritzen et al., 1971; Baulieu and Jung, 1970) and from prostatic nuclei in association with a 3S protein (Fang et al., 1969). As well, a second cytosol receptor has been observed in some systems (Mainwaring, 1969; Baulieu and Jung, 1970) with a sedimentation coefficient of 8S. Sedimentation analysis of nuclear and cytoplasmic extracts after *in vitro* incubations with radioactive dihydrotestosterone has also demonstrated the presence of these receptor proteins (Fang et al., 1969; Baulieu and Jung, 1970). Although the above studies indicated that one or possibly two different dihydrotestosterone binding proteins are present in prostatic cytosol, it was neither established whether the receptors in the 3S and 8S fractions were related, nor alternatively, whether there were several types of binding proteins with common sedimentation properties. Furthermore, because of similarities in migration rates in sucrose density gradients, the 3-4S cytoplasmic binding protein and the 3-4S nuclear binding protein were believed to be identical but no information was available as to their homogeneity in other analytical systems.

Accordingly, the experiments described in this Chapter were undertaken to determine the number of binding proteins in the cell cytoplasm and nucleus, and to study the relationship between cytoplasmic and nuclear receptors in greater detail. Toward this end the principal analyses were carried out using column chromatography procedures.

B. Materials and Methods

1. Conditions for In Vitro Incubations

Nuclear and cytoplasmic fractions were isolated in the manner described in Chapter II. Approximately 5 x 10^7 nuclei, suspended in 1 ml of Tris-EDTA buffer containing 50 mM NaCl, were incubated at 25°C for 90 min with the appropriate radioisotope. Following incubation, the sample was sonicated and extracted with high molar salt (final concentration 0.6 M). Where indicated, the sonication and salt extraction were performed prior to incubation.

Cytosol protein from 1 g of prostatic tissue was incubated with ^{3}H-steroid at 25° C for 90 min and then precipitated with ammonium sulphate. The sample was saturated to 80% with ammonium sulphate which was added slowly over a 1 hour period while the temperature was controlled at 4° C. Precipitated protein was collected by centrifugation at 17,000 x g for 20 min in a Sorvall centrifuge and suspended in 1 ml of Tris-EDTA buffer, pH 7.0. Any departures from this procedure are indicated in the text.

2. Column Chromatography Procedures

Samples were desalted by passage through Sephadex G-25 columns (1 cm x 40 cm) prior to application to cellulose phosphate columns. Proteins were eluted from the columns (1 cm x 15 cm) with an ionic gradient (0 to 0.8 M NaCl) and fractions of 4.2 ml were collected.

Sephadex G-200 analysis of extracts was performed on 1 cm x 90 cm columns in which Tris-EDTA buffer, pH 7.0, containing either no NaCl or 0.6 M NaCl, served as the elution buffer.

C. Results

PART I - Cytosol Receptors

1. Precipitation of Cytosol Proteins with Ammonium Sulphate

As a result of the fractionation and isolation procedures, cytosol preparations from 1 g of prostatic tissue usually had a final volume of 10 ml. While the protein concentration in these preparations was adequate for the binding studies described in Chapter III, the relatively large volume was cumbersome for further analysis of the steroid-protein complexes. To overcome this problem, the cytosol proteins were concentrated by precipitation with ammonium sulphate.

Rats castrated 24 hours previously, were used in these experiments. This interval of castration gives rise to maximal binding of dihydrotestosterone (Fig. 3.6) since most of the endogenous steroids have been cleared from the ventral prostate (Fang et al., 1969). Cytosol from 1 g of prostatic tissue (the pooled prostates of 3 to 4 rats) was incubated with 0.8 μCi of either [1,2-^{3}H]testosterone or [1,2-^{3}H]dihydrotestosterone at 25° C for 90 min. After the incubation period, each sample was divided into 3 equal volumes. Two of these were brought to 50% and 80% saturation with ammonium sulphate and the remaining one served as control. The precipitated protein was collected by centrifugation, resuspended in 1 ml of Tris-EDTA buffer, pH 7.0, and subjected to gel filtration on Sephadex G-25. The results of these experiments are shown in Table 4.1. Virtually all of the radioactivity associated with the receptor proteins was recovered in the 80% ammonium sulphate precipitate. When portions of the 80% supernatant were assayed for protein bound radioactivity, no binding was detected. Thus ammonium sulphate precipitation provided a simple and quick method for concentrating cytosol proteins.

2. Chromatography of Cytosol Protein on Cellulose Phosphate

Partial purification of steroid binding proteins in prostatic cytosol was undertaken using cellulose phosphate. Rats, castrated 24 hours previously, were sacrificed and the cytosol fraction from 1 g of prostatic tissue was isolated. The cytosol protein was incubated at 25° C for 90 min with either [1,2-^{3}H]dihydrotestosterone or [1,2-^{3}H]testosterone at a concentration of 1 x 10^{-9} M.

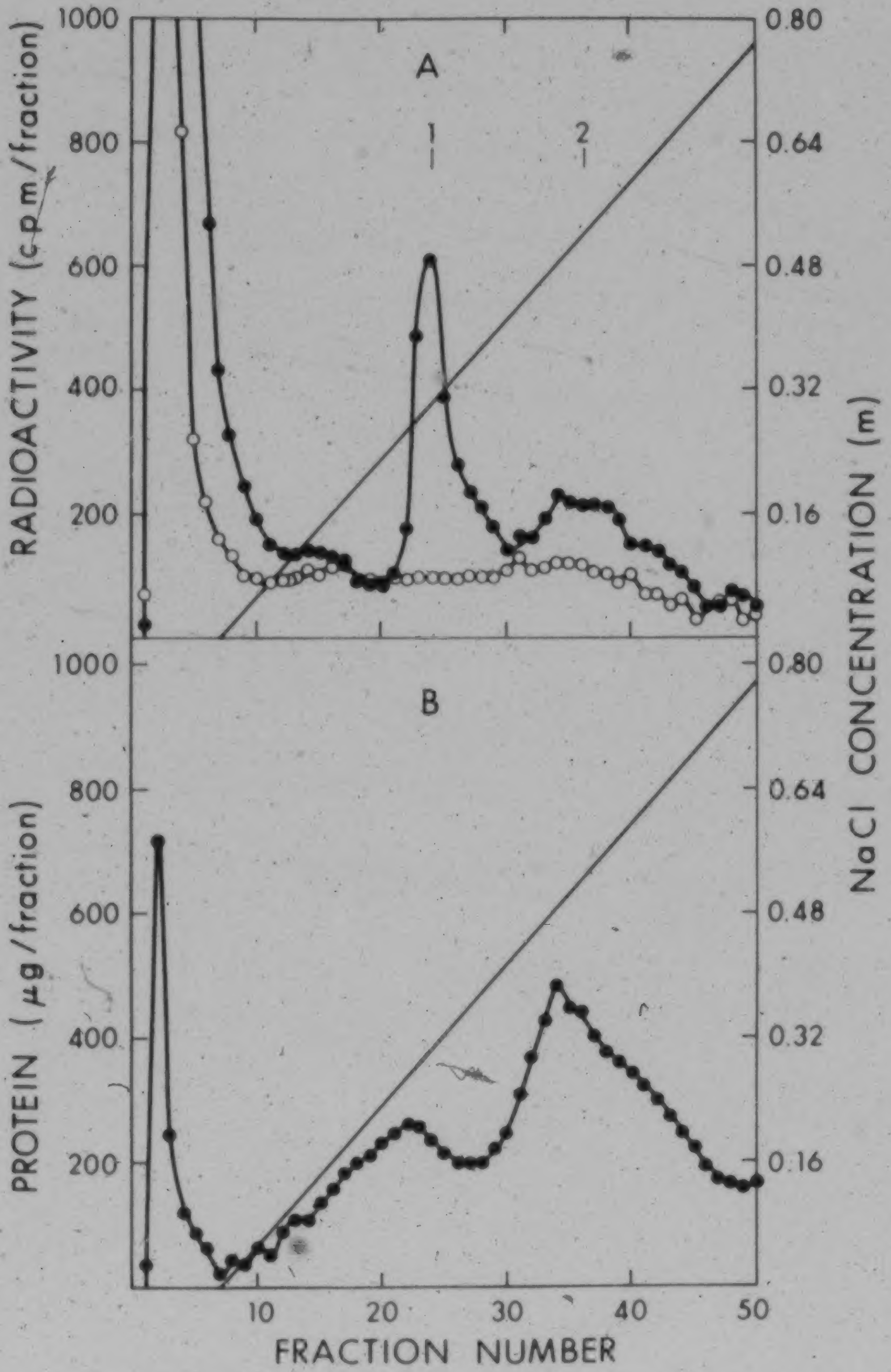
TABLE 4.1

PROTEIN PRECIPITATION WITH AMMONIUM SULPHATE

% $(NH_4)_2SO_4$	Radioactivity Bound (dpm/g Wet Weight x 10^{-3})	
	Testosterone	Dihydrotestosterone
0 (Control)	6.1 (100%)	13.0 (100%)
50	4.5 (74%)	8.4 (65%)
80	5.9 (97%)	12.2 (94%)

Cytosol protein from 1 g of prostatic tissue was incubated with either 0.8 µCi of [1,2-^{3}H]dihydrotestosterone or [1,2-^{3}H]testosterone under standard conditions (i.e. 25° C for 90 min). On completion of incubation, the samples were divided into 3 equal portions and made to 5 ml each with 0.25 M sucrose solution. Solid ammonium sulphate was added over a period of 1 hr to give a final concentration of 50% or 80% saturation at 0° C. The protein precipitates were collected by centrifugation, resuspended in 1 ml of Tris-EDTA buffer, pH 7.0, and analyzed for androgen binding by gel filtration. Binding in the control sample was determined directly by passing the unprecipitated cytosol through the column.

Fig. 4.1. Demonstration of in vitro cytosol receptor peaks on cellulose phosphate. Cytosol preparations were obtained from male rats castrated 24 hours previously. An amount of cytosol protein equivalent to 1 g of prostate was incubated at 25° C for 90 min in 10 ml Tris-EDTA buffer, pH 7.0 containing 0.25 M sucrose and 1.0 x 10^{-9} M radioactive steroid. Following the incubation the charged cytosol was precipitated with ammonium sulphate (80% saturation). The precipitate was desalted by passage through Sephadex G-25 gel and then applied to a 1 cm x 15 cm column of cellulose phosphate. Proteins were eluted with an ionic gradient consisting of Tris-EDTA buffer, pH 7.0 containing 0 to 0.8 M NaCl. Fractions of 4.2 ml each were collected, the O.D. at 260 and 280 nm and the radioactivity in each fraction were measured. Panel A, radioactivity recovered; ○——○ [1,2-^{3}H]testosterone; ●——● [1,2-^{3}H]dihydrotestosterone. Panel B, typical profile of protein distribution.


A
1
2
B
RADIOACTIVITY (c.p.m/fraction)
PROTEIN (μg/fraction)
NaCl CONCENTRATION (m)
FRACTION NUMBER
1000
800
600
400
200
0.80
0.64
0.48
0.32
0.16
10
20
30
40
50

The charged cytosol protein was precipitated with ammonium sulphate at 80% saturation. The precipitate was resuspended in 1 ml of Tris-EDTA buffer, pH 7.0, and then desalted by gel filtration through Sephadex G-25. The sample recovered in the void volume was applied to a column of cellulose phosphate. Proteins were eluted with an ionic gradient and the radioactivity in each fraction was measured. It is evident from the results shown in Fig. 4.1 that while [1,2-^{3}H]-testosterone was spread diffusely throughout fractions 10 to 50, [1,2-^{3}H]dihydrotestosterone was recovered in two peaks which corresponded closely with the two peaks of eluted protein. Most of the radioactivity was recovered in the Peak 1 area. When the run-through fractions from these columns (i.e. fractions 1 to 7 of Fig. 4.1) were examined on Sephadex G-25 columns no protein bound radioactivity was detected.

There were no qualitative differences in the cellulose phosphate chromatograms obtained when the ammonium sulphate precipitation step, and the subsequent desalting procedure, were deleted from the experimental design. Also, when cytosol protein from intact rats was used, the radioactive profile was similar to that shown in Fig. 4.1. Neither testosterone nor dihydrotestosterone by themselves are retained by cellulose phosphate.

3. Chromatography of Cytosol Protein on Sephadex G-200

Experiments were next carried out to investigate the behaviour of steroid-receptors on Sephadex G-200 columns. Cytosol protein from castrate rats was incubated as before with either [1,2-^{3}H]testosterone or [1,2-^{3}H]dihydrotestosterone, precipitated with ammonium sulphate, and applied to a Sephadex column. Proteins were eluted with Tris-EDTA buffer, pH 7.0, or Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The results shown in Fig. 4.2 and 4.3 give the protein and radioisotope distributions obtained after experiments with dihydrotestosterone and testosterone respectively.

When NaCl was excluded from the eluant, 2 peaks of radioactivity (i.e. Peaks I, III) were observed in the experiments with dihydrotestosterone (Fig. 4.2A); in the presence of NaCl, 3 radioactive peaks were present (Fig. 4.2B). The absence of Peak II label and the relatively greater proportion of radioactivity associated with Peak I under conditions of low ionic strength suggests that Peak I may represent an aggregated form of steroid-receptor. In both the absence and presence of NaCl, the greatest amount of radioactivity was recovered in Peak III.

When similar experiments were done using [1,2-^{3}H]-testosterone as substrate, two peaks of radioactivity were detected (Fig. 4.3). Both peaks were eluted in volumes corresponding to Peak I and Peak III observed in experiments with dihydrotestosterone. However, the amount of label associated with these peaks was considerably less when compared to the amount of binding obtained with dihydro-

Fig. 4.2. Demonstration of *in vitro* cytosol receptor peaks for dihydrotestosterone on Sephadex G-200. Cytosol protein from 1 g of prostate from castrated rats, was incubated at 25° C for 90 min in 10 ml of 0.25 M sucrose solution containing radioactive dihydrotestosterone (1×10^{-9} M). After the incubation period the protein was precipitated with ammonium sulphate (80% saturation) and collected by centrifugation. The precipitate was then resuspended in 1 ml of Tris-EDTA buffer, pH 7.0, and applied to a Sephadex G-200 column (1 cm x 90 cm). Proteins were eluted either with NaCl-free buffer (Panel A) or with buffer containing 0.6 M NaCl (Panel B). Fractions of 1.5 ml each were collected; the absorbance at 260 and 280 nm and the radioactivity in each fraction were determined. Radioactivity, ●——● ; protein, ○——○ .

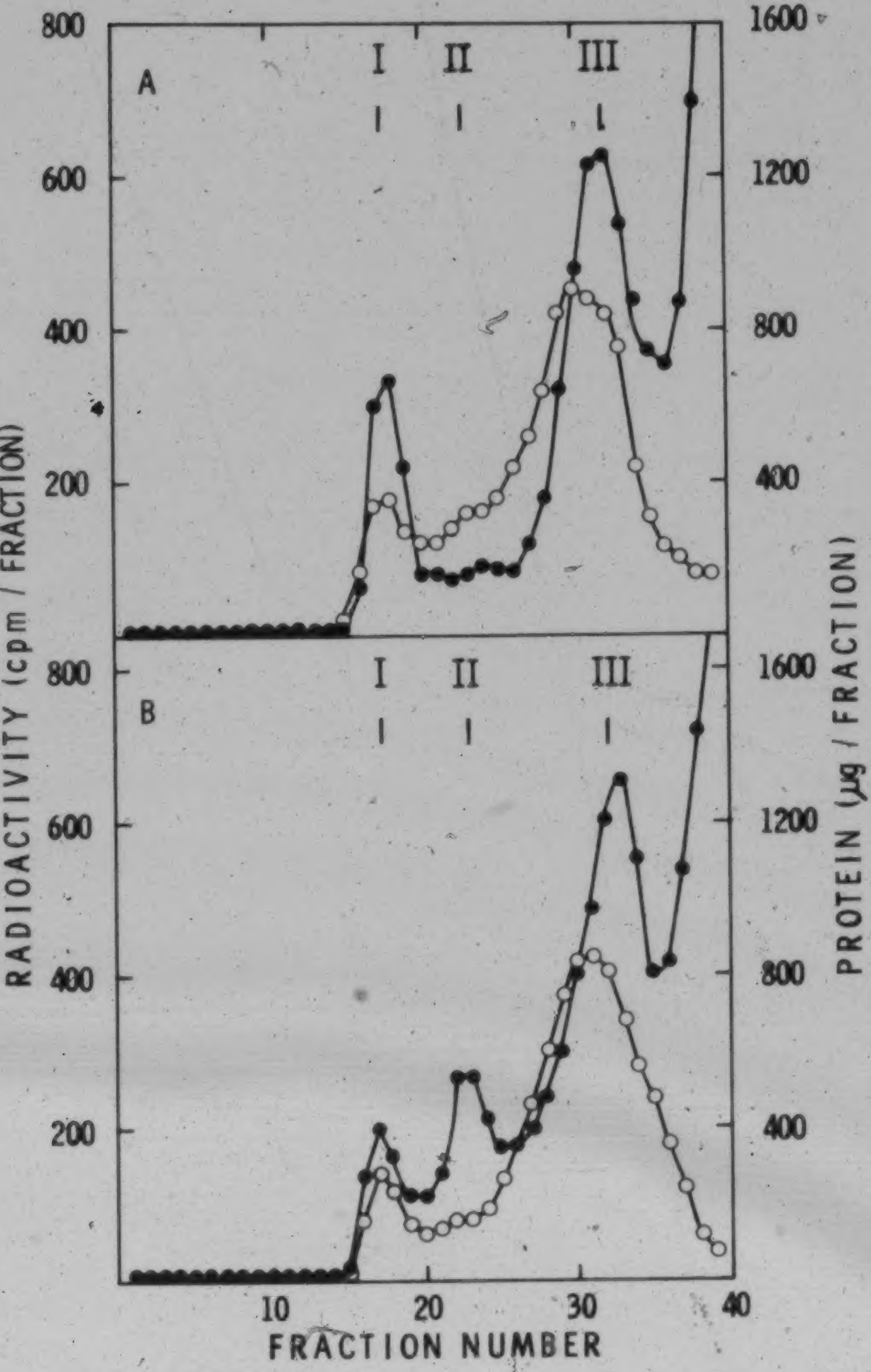
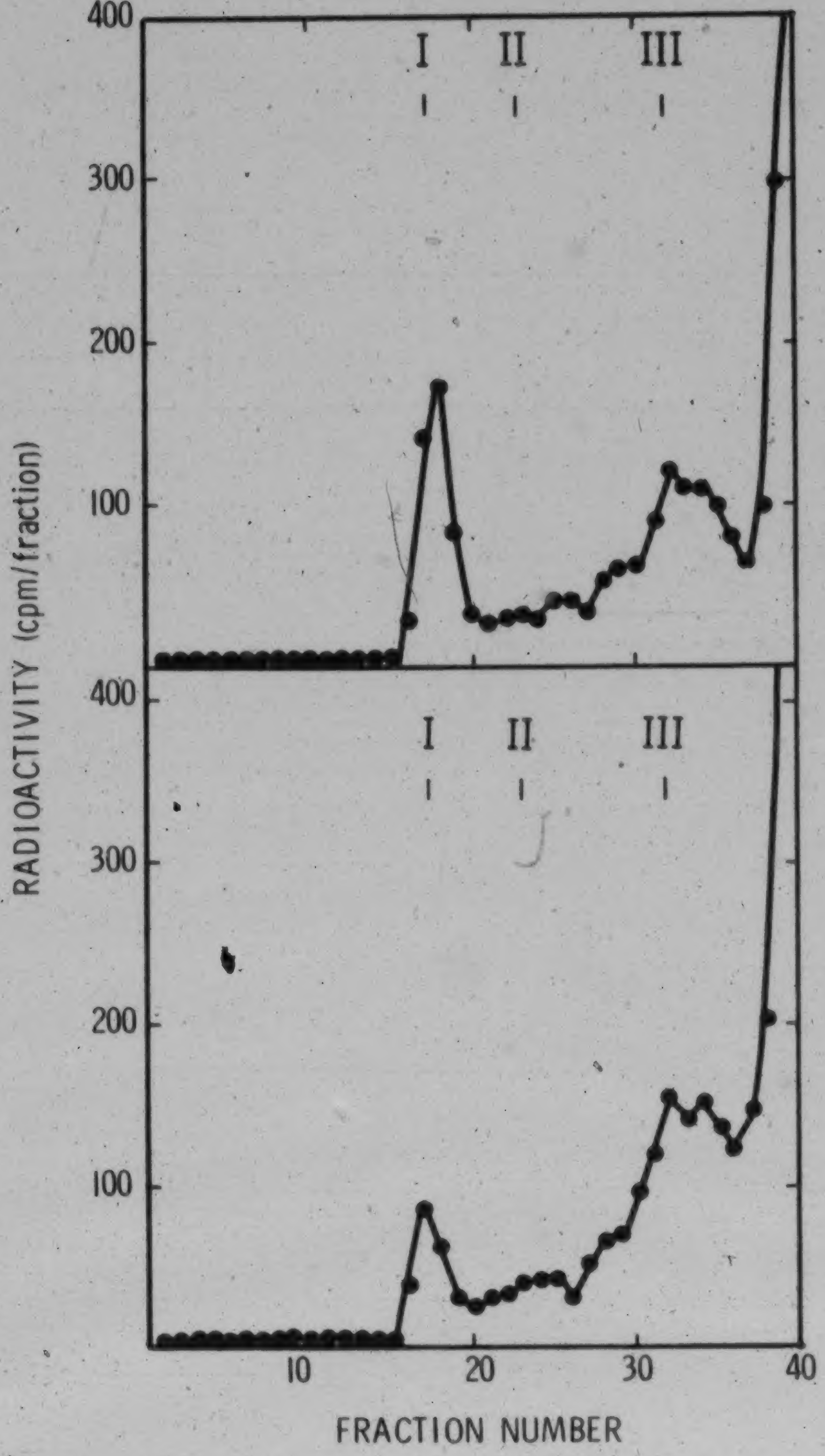

A
B
I
II
III
RADIOACTIVITY (cpm / FRACTION)
PROTEIN (μg / FRACTION)
FRACTION NUMBER
800
600
400
200
1600
1200
800
400
10
20
30
40

Fig. 4.3. Demonstration of in vitro cytosol receptor peaks for testosterone on Sephadex G-200. Prostatic cytosol from castrate rats was incubated and analyzed on Sephadex G-200 in an identical manner to that described in Fig. 4.2. 10 ml of cytosol protein from 1 g of prostate was incubated with [1,2-^{3}H]testosterone (1 x 10^{-9} M). Proteins were eluted with Tris-EDTA buffer (Panel A) or with Tris-EDTA buffer containing 0.6 M NaCl (Panel B). Fractions of 1.5 ml each were collected and the radioactivity in each was determined.


400
300
200
100
I
II
III
400
300
200
100
I
II
III
RADIOACTIVITY (cpm/fraction)
10
20
30
40
FRACTION NUMBER

testosterone. A second difference in the results was that testosterone failed to bind to protein corresponding to the Peak II area of the chromatogram even in the presence of 0.6 M NaCl (Fig. 4.3B).

4. Repeat Chromatography of Peaks I, II and III on Sephadex G-200

Since the results shown in Fig. 4.2 suggested that part of the [1,2-^{3}H]dihydrotestosterone associated with Peak I arose from aggregation of Peak II steroid-receptors, experiments were conducted in which Peak I was isolated and then rechromatographed on Sephadex G-200 under conditions of high ionic strength. Specifically, cytosol from 1 g of prostatic tissue was incubated with [1,2-^{3}H]-dihydrotestosterone, precipitated with ammonium sulphate, and applied to a Sephadex G-200 column. Proteins were eluted from the column with Tris-EDTA buffer (no NaCl), pH 7.0, and fractions 17 to 19 were pooled (Peak I). In order to ensure that sufficient radioactivity was bound to this protein, the sample was again incubated at 25° C for 90 min with [1,2-^{3}H]dihydrotestosterone. The Peak I protein was then precipitated with ammonium sulphate and run on a Sephadex G-200 column in which Tris-EDTA buffer containing 0.6 M NaCl was used as the elution buffer. It is evident from the results shown in Fig. 4.4 that almost all of the radioactivity was recovered in the Peak I area of the chromatogram. If Peak I was formed as a result of reversible aggregation of Peak II receptors, disaggregation of the former should have occurred with a corresponding increase in the latter when 0.6 M

Fig. 4.4. Repeat chromatography of Sephadex Peak I on Sephadex G-200. Fractions 17 to 19 (Fig. 4.2A) were pooled and incubated at 25° C for 90 min with 1 x 10^{-11} moles of [[illegible]2-^{3}H]dihydrotestosterone. The protein was precipitated with ammonium sulphate and applied to a Sephadex G-200 column. Fractions of 1.3 ml each were eluted with buffer containing 0.6 M NaCl, and the radioactivity and protein in each was measured. Radioactivity, ●———● ; protein, ○———○ .

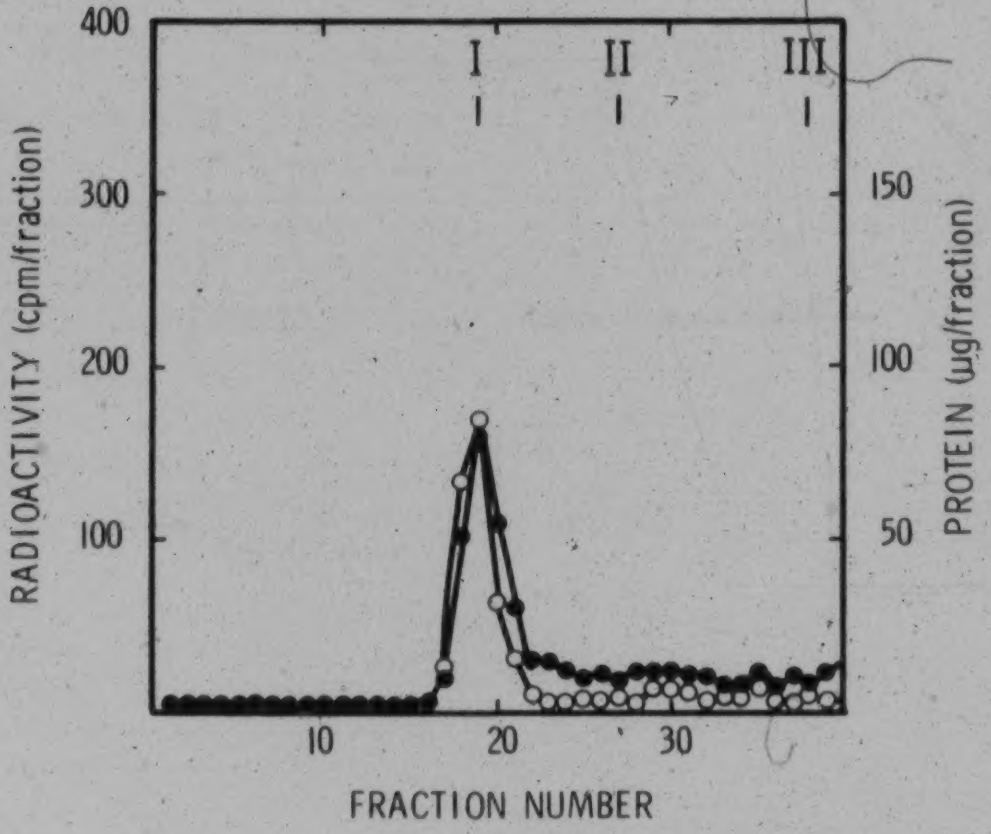
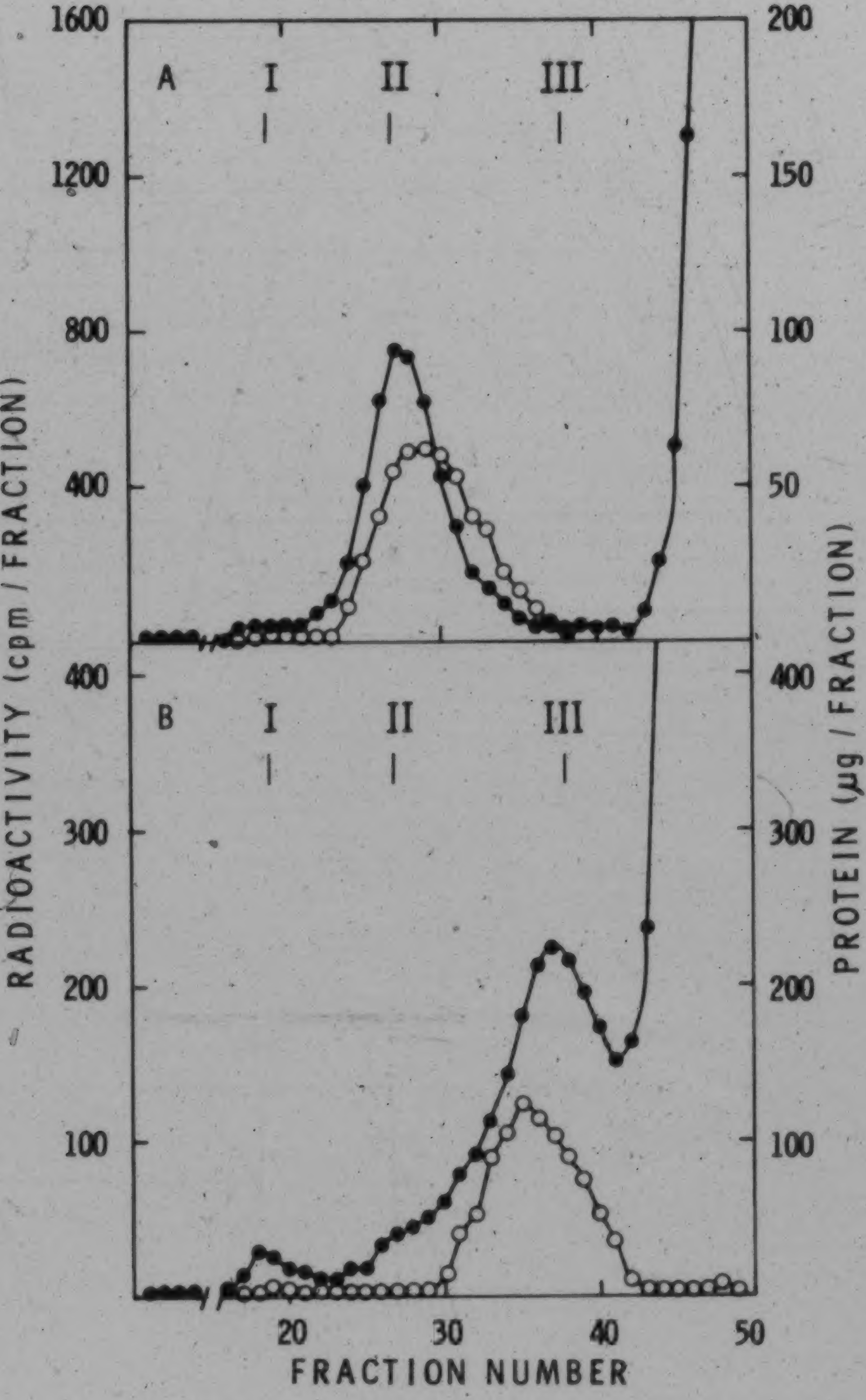

400
300
200
100
RADIOACTIVITY (cpm/fraction)
150
100
50
PROTEIN (µg/fraction)
I
II
III
10
20
30
FRACTION NUMBER

Fig. 4.5. Repeat chromatography of Sephadex Peaks II and III on Sephadex G-200. Fractions 21 to 24 (Peak II) and 31 to 34 (Peak III) from the experiment shown in Fig. 4.2B were pooled and reincubated with 1 x 10^{-11} moles of [1,2-^{3}H]dihydrotestosterone. The samples were precipitated with ammonium sulphate (80% saturation) and applied to a Sephadex G-200 column. Fractions of 1.3 ml were eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl and the radioactivity and protein in each were measured. Panel A, repeat chromatography of Peak II. Panel B, repeat chromatography of Peak III. Radioactivity, ●——● ; protein ○——○ .


A
B
I
II
III
1600
1200
800
400
300
200
100
200
150
100
50
400
300
200
100
20
30
40
50
RADIOACTIVITY (cpm / FRACTION)
PROTEIN (µg / FRACTION)
FRACTION NUMBER

NaCl was included in the eluant. However, this was not the case. While the data are consistent with the idea that Peak I radioactivity arises as a result of irreversible aggregation of smaller steroid receptors, it is equally possible that the experimental manipulations damaged the protein in such a way as to prevent dissociation of the aggregates.

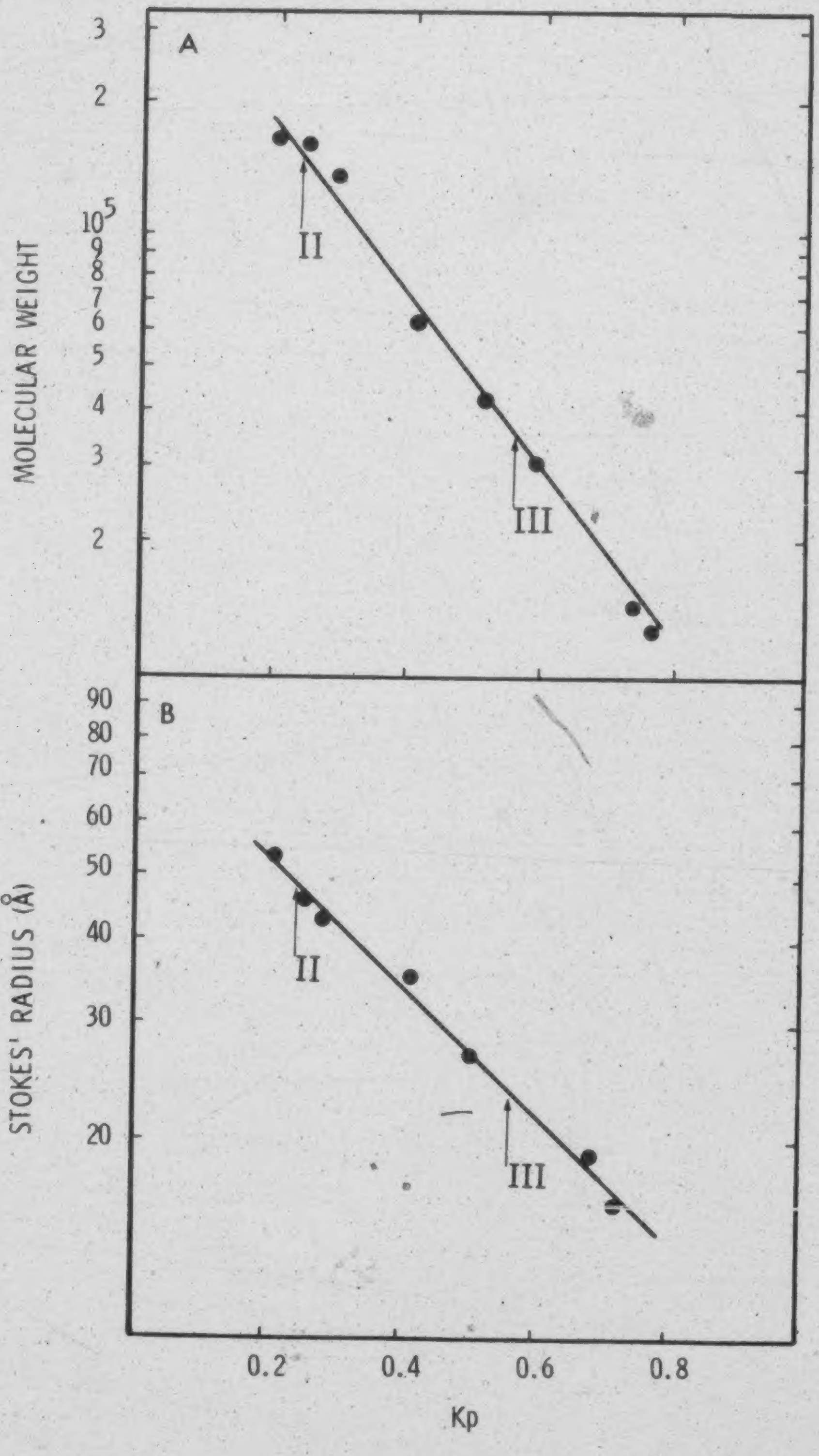
Experiments similar to those described above with Peak I proteins were performed with Peak II and Peak III material. Fig. 4.5 shows the results of experiments in which Peak II receptors (Panel A) or Peak III receptors (Panel B) were isolated, incubated with ^{3}H-steroid, and again chromatographed on Sephadex G-200 columns. In both instances the major portion of the protein associated radioactivity was recovered in the same fractions that were applied to the columns.

5. Stokes' Radii and Molecular Weights

Gel filtration provides a rapid and simple method for estimating the molecular weight and molecular size of proteins. Several studies (Whitaker, 1963; Andrews, 1964, 1965) have shown that the elution volumes of proteins are largely determined by their molecular weights. Molecular weights can be estimated in relatively crude preparations and hence extensive purification is not required. However, when molecular weights are determined by gel filtration, calibration substances must be from the same homologous group of proteins having a related physical form or symmetry. Since no information is available as to the molecular shape of the cytoplasmic androgen receptors, estimates of the molecular weights of these proteins, using globular proteins as standards, may be inaccurate. However, the derivation of Stokes' radii from Sephadex elution data without assuming a particular molecular shape is acceptable (Siegel and Monty, 1966; Andrews, 1970).

A Sephadex G-200 column (1 cm x 90 cm) was calibrated with 200 to 500 µg of each of the following standards; cytochrome c, RNase I, DNase, ovalbumin, bovine serum albumin and its dimer, aldolase, and bovine γ globulin. Calculation of the partition coefficient (K_p) between the liquid phase and the gel phase was done using the relationship (Laurent and Killander, 1964) $K_p = (V_e - V_o)/V_T - V_o)$, where V_e is the elution volume of the particular solute, V_T is the total volume of the gel bed, and V_o is the void volume. When the K_p values obtained for the protein standards were plotted against their respective molecular weights (Fig. 4.6A) or Stokes' radii (Fig. 4.6B) on semi-log scale, a linear relationship was observed in each case. From these plots it was possible to calculate the desired physical parameters of the cytoplasmic receptor proteins for dihydrotestosterone. For example, Peak II protein had an apparent molecular weight of 147,000 - 153,000 and a Stokes' radius of 47 - 49 Å, while Peak III protein had values of 34,000 - 36,000 and 23 - 25 Å for molecular weight and Stokes' radius respectively. Because Peak I protein appeared in the void volume, estimates of molecular size could not be calculated for this receptor. The relevance of the molecular

Fig. 4.6. Correlation of Kp with molecular weight and Stokes' radius. Partition coefficients (Kp) of protein standards were computed from data obtained from Sephadex G-200 experiments according to the relationship. $Kp = \frac{V_e - V_o}{V_T - V_o}$. The arrows indicate the Kp values obtained for Peak II and Peak III complexes. The relationship between partition coefficients and molecular weights and Stokes' radii are shown in Panel A and Panel B respectively.


A
MOLECULAR WEIGHT
3
2
10^5
9
8
7
6
5
4
3
2
II
III
B
STOKES' RADIUS (Å)
90
80
70
60
50
40
30
20
II
III
0.2
0.4
0.6
0.8
Kp

weights ascribed to Peak II and Peak III proteins are of course contingent on accuracy of the assumption that both proteins have a globular shape.

6. Sequential Analysis of Cytosol Proteins on Cellulose Phosphate and Sephadex G-200 Columns

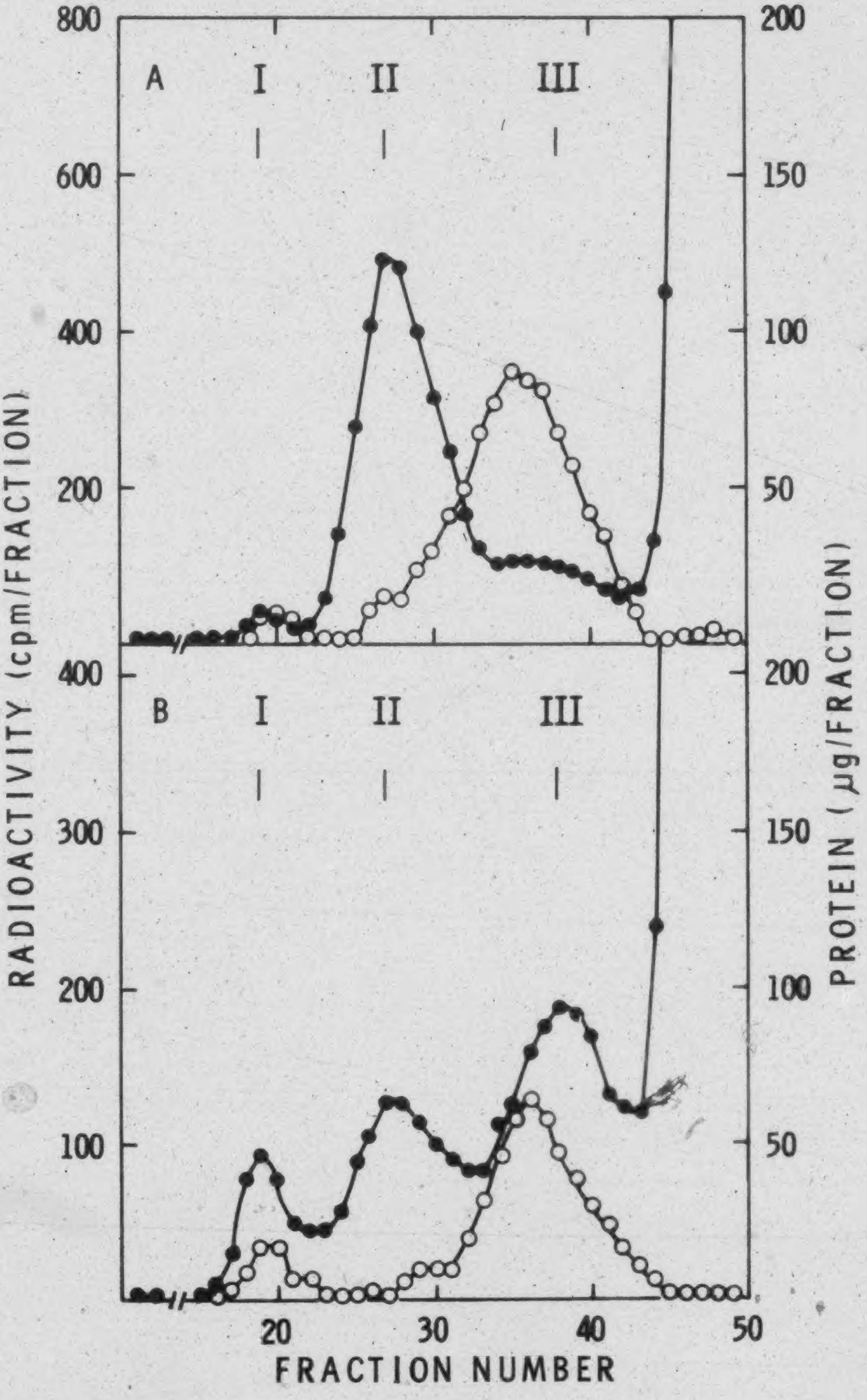
Although one can detect at least two distinct types of dihydrotestosterone receptor proteins using Sephadex G-200 chromatography (Fig. 4.2) and cellulose phosphate chromatography (Fig. 4.1), it is not clear whether these proteins are equivalent in the different analytical systems. For example, does Sephadex Peak III receptor correspond to cellulose phosphate Peak 1 receptor, and similarly is Sephadex Peak II receptor the same as cellulose phosphate Peak 2 receptor? To answer these questions and to provide further information as to the number of discrete androgen binding proteins present in prostatic cytoplasm, a two step purification procedure was employed.

Ten ml of cytosol protein from castrated rats was incubated as before in the presence of 1×10^{-9} M [1,2-^{3}H]dihydrotestosterone. The labelled protein was precipitated with ammonium sulphate, desalted on a Sephadex G-25 column, and applied to a cellulose phosphate column. After elution with a salt gradient, fractions containing Peak 1 receptor (i.e. fractions corresponding to tubes 24 to 27 of the experiment shown in Fig. 4.1) were pooled and then reincubated at 25° C for 90 min with [1,2-^{3}H]dihydrotestosterone.

On completion of the second incubation, the sample was again precipitated with ammonium sulphate and subsequently run on a Sephadex G-200 column. The results of this experiment are shown in Fig. 4.7A. Virtually all of the dihydrotestosterone binding activity associated with cellulose phosphate Peak 1 was eluted from the Sephadex G-200 column in the same position as Sephadex Peak II receptors. By analogy, therefore, one might expect that cellulose phosphate Peak 2 is equivalent to the Sephadex Peak III.

In order to examine this possibility, Peak 2 protein was isolated as described in the experiment above using cellulose phosphate (fractions 34 to 38 in Fig. 4.1) and then reincubated with [1,2-^{3}H]dihydrotestosterone. Following incubation, the sample was precipitated with ammonium sulphate and then chromatographed on Sephadex G-200. Unexpectedly, when the radioisotope and protein concentrations were measured in the eluted fractions, 3 peaks of radioactivity were found corresponding to Peaks I, II, and III (Fig. 4.7B). Since cellulose phosphate Peaks 1 and 2 were well separated, the possibility that Peak 2 protein was contaminated by Peak 1 protein prior to chromatography on Sephadex is remote. Thus, it would appear that there are several forms of receptors for dihydrotestosterone in prostatic cytosol. Three have identical affinity for cellulose phosphate but possess Stokes' radii of approximately 24 Å, 48 Å and greater than 48 Å (Sephadex Peak I). A fourth form of receptor has a Stokes' radius of about 48 Å but displays a reduced affinity for cellulose phosphate.

Fig. 4.7. Chromatography of cellulose phosphate Peaks 1 and 2 on Sephadex G-200. Peak 1 and Peak 2 complexes were isolated from cellulose phosphate experiments with *in vitro* labelled cytosol receptors. Fractions corresponding to Peak 1 protein were pooled, incubated with 2×10^{-11} moles of [1,2-^{3}H]dihydrotestosterone, precipitated with ammonium sulphate, and then applied to a Sephadex G-200 column. Similar operations were performed with Peak 2 protein fractions. In each case the Sephadex columns were eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl, and fractions of 1.3 ml were collected. Panel A, gel filtration of Peak 1. Panel B, gel filtration of Peak 2. Radioactivity, ●——● ; protein ○——○ .

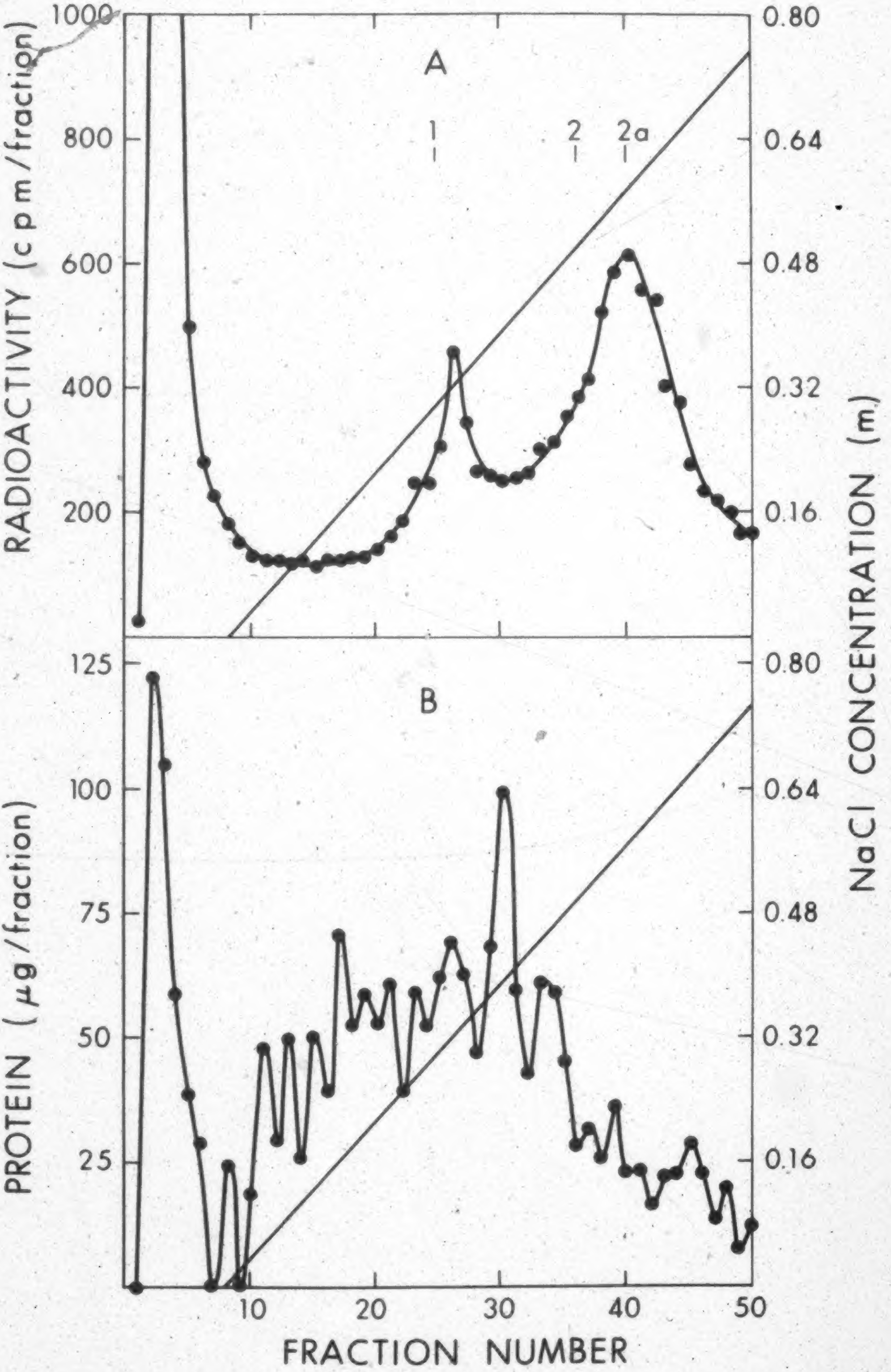

A
B
I
II
III
800
600
400
300
200
100
150
50
RADIOACTIVITY (cpm/FRACTION)
PROTEIN (μg/FRACTION)
20
30
40
50
FRACTION NUMBER

PART II - Nuclear Receptors

1. Chromatography of Nuclear Extracts on Cellulose Phosphate

Partial purification of intranuclear steroid-receptors was undertaken next and the first approach involved the use of cellulose phosphate. Intact animals were used in these experiments since it was assumed that there would be more receptor sites in prostatic nuclei of normal rats compared to nuclei of castrated rats (Fig. 3.6). Purified nuclei were incubated in Tris-EDTA buffer, pH 7.0, containing 0.05 M NaCl, and 1×10^{-11} moles of [1,2-^{3}H]dihydrotestosterone at 25° C for 90 min. Following incubation, the nuclei were sonicated, and extracted with buffer, containing 0.6 M NaCl. The resulting nuclear extract was desalted by gel filtration and then applied to a cellulose phosphate column. The column was eluted with an ionic gradient, and fractions were assayed for protein and radioactivity. Two radioactive peaks were observed as shown in Fig. 4.8. While one peak appeared in fractions corresponding to Peak 1 obtained in experiments with cytosol (Fig. 4.1), there was less radioactivity in this peak than in the second which was eluted at approximately 0.6 M NaCl. Inspection of Figs. 4.1 and 4.8 reveals that the second nuclear peak is not identical with cytosol Peak 2 (eluted at 0.52 M NaCl) and accordingly is depicted as Peak 2a. If the nuclei were first extracted and then incubated with [1,2-^{3}H]dihydrotestosterone, the profile of radioactivity obtained did not differ from that shown in Fig. 4.8.

Fig. 4.8. Demonstration of *in vitro* nuclear receptor peaks on cellulose phosphate. Prostatic nuclei were obtained from intact male rats according to the isolation procedures described under Methods. Approximately 5.0×10^7 nuclei were incubated at 25° C for 90 min in 1 to 2 ml Tris-EDTA buffer, pH 7.0 containing 1×10^{-11} moles of [1,2-^{3}H]-dihydrotestosterone. Following the incubation period, the nuclei were sonicated and extracted with Tris-EDTA buffer, pH 7.0 containing 0.6 M NaCl. The nuclear extract was desalted by gel filtration, applied to a 1 cm x 15 cm column of cellulose phosphate, and proteins were eluted with an ionic gradient consisting of Tris-EDTA buffer, pH 7.0 containing 0 to 0.8 M NaCl. Fractions of 4.2 ml each were collected and the absorbance and the radioactivity in each fraction were measured. Panel A, radioactivity recovered; ●——● [1,2-^{3}H]dihydrotestosterone. Panel B, typical profile of protein distribution.


A
B
1
2
2a
RADIOACTIVITY (c p m / fraction)
1000
800
600
400
200
PROTEIN (µg / fraction)
125
100
75
50
25
NaCl CONCENTRATION (m)
0.80
0.64
0.48
0.32
0.16
10
20
30
40
50
FRACTION NUMBER

Since it was shown previously that there is a decrease in the levels of intranuclear binding of dihydrotestosterone in castrated animals (Fig. 3.6), it was of interest to determine whether this effect was due to a specific reduction in either Peak 1 or Peak 2a binding. Accordingly, nuclear extracts obtained from rats castrated 24 hours previously were incubated and chromatographed on cellulose phosphate in the same manner described above for preparations from intact animals. The results shown in Fig. 4.9 reveal that in these experiments [1,2-^{3}H]dihydrotestosterone was bound almost exclusively to Peak 1 protein. Very little radioactivity was associated with the Peak 2a area of the chromatogram. Apparently, castration causes a selective loss of Peak 2a receptor sites.

2. Chromatography of Extracts of Nuclei on Sephadex G-200

The second approach to the partial purification of nuclear receptors involved the use of Sephadex G-200. Nuclear extracts, obtained from the prostates of intact and castrated rats, were incubated with [1,2-^{3}H]dihydrotestosterone for 90 min at 25° C and then applied directly to a column of Sephadex G-200. Elution of the proteins was accomplished with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. Figs. 4.10A and 4.10B show the distribution of radioactivity and optical density at 260 nm observed with nuclear extracts from intact and castrated rats respectively. In the former case two distinct peaks of radioactivity were present - one associated with the void volume, and the other with an elution volume

Fig. 4.9. Chromatography of extracts of prostatic nuclei of castrated rats on cellulose phosphate. A nuclear extract, equivalent to 5 x 10^7 nuclei, was obtained from rats castrated 24 hours previously. The sample (1 to 2 ml) was incubated at 25° C for 90 min with 1 x 10^{-11} moles of [1,2-^{3}H]dihydrotestosterone, desalted by gel filtration, and then chromatographed on cellulose phosphate as described in the legend to Fig. 4.8. Fractions of 4.2 ml were collected and the radioactivity in each was determined.

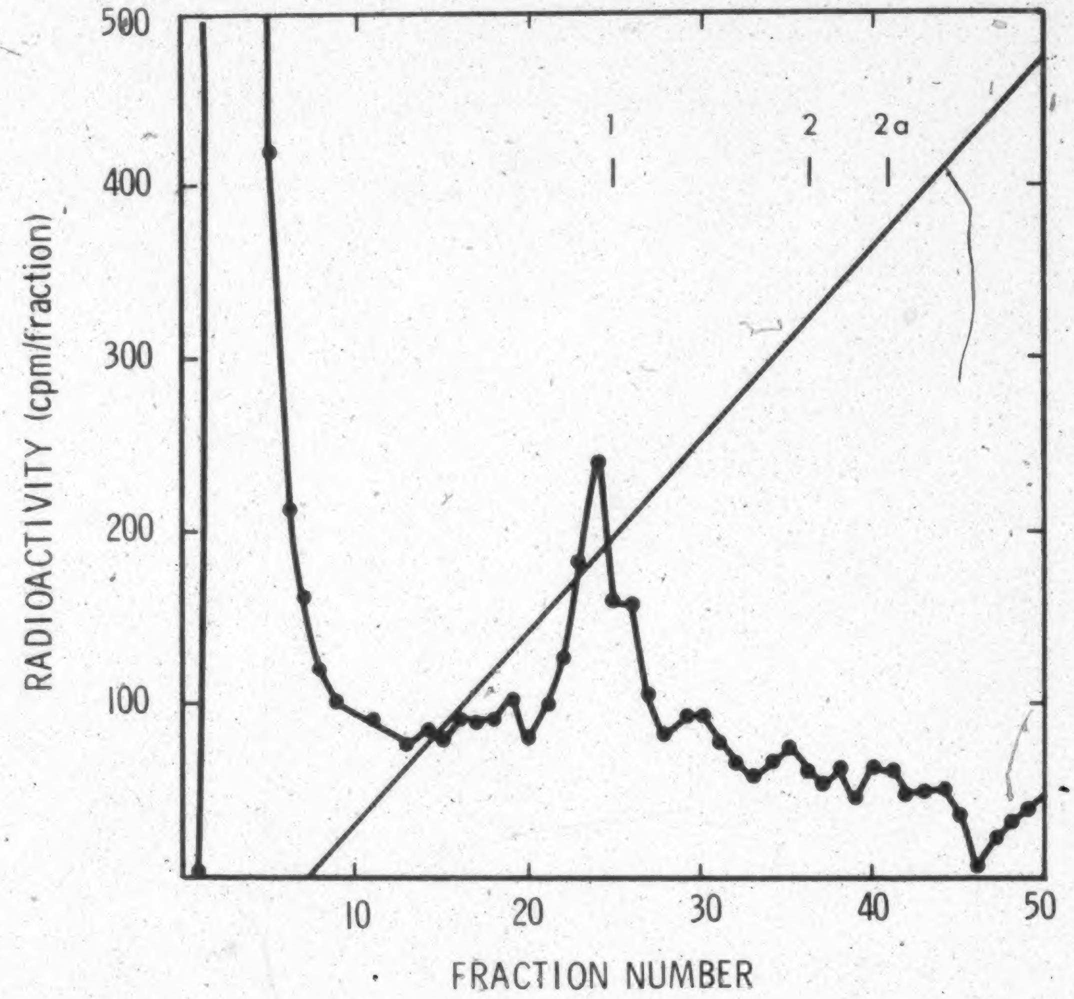
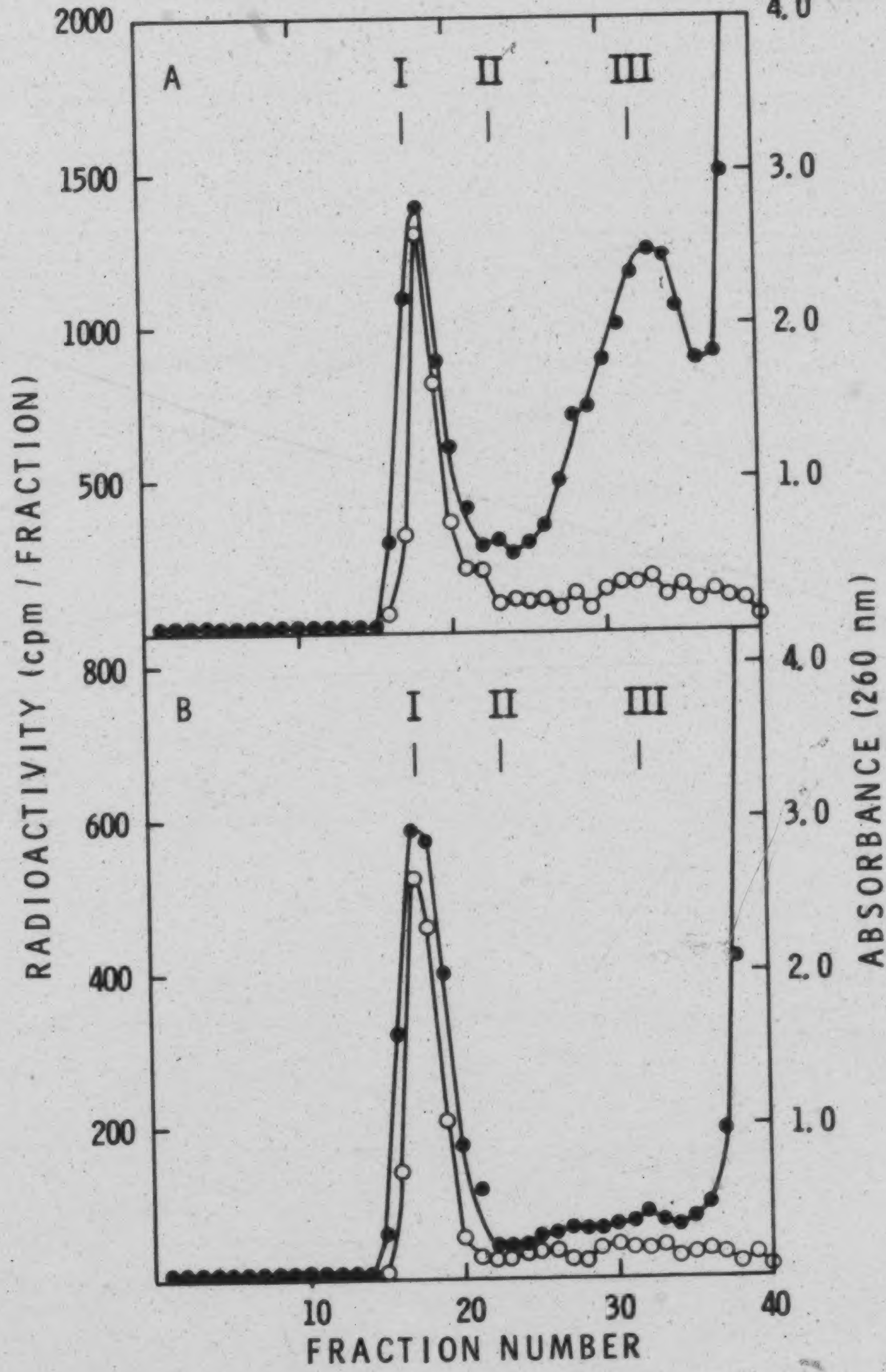

RADIOACTIVITY (cpm/fraction)
500
400
300
200
100
10
20
30
40
50
FRACTION NUMBER
1
2
2a

Fig. 4.10. Demonstration of *in vitro* nuclear receptor peaks on Sephadex G-200. Approximately 5×10^7 nuclei were isolated from intact and castrated rats and extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The nuclear extracts (1 to 2 ml) were incubated with 1×10^{-11} moles of $[1,2\text{-}^{3}H]$dihydrotestosterone for 90 min at 25° C and then applied to a Sephadex G-200 column. Fractions of 1.5 ml were eluted from the column with Tris-EDTA buffer containing 0.6 M NaCl. Panel A, extracts prepared from prostatic nuclei of intact rats. Panel B, extracts prepared from prostatic nuclei of castrated rats. Radioactivity, ●——● ; absorbance ○——○ .

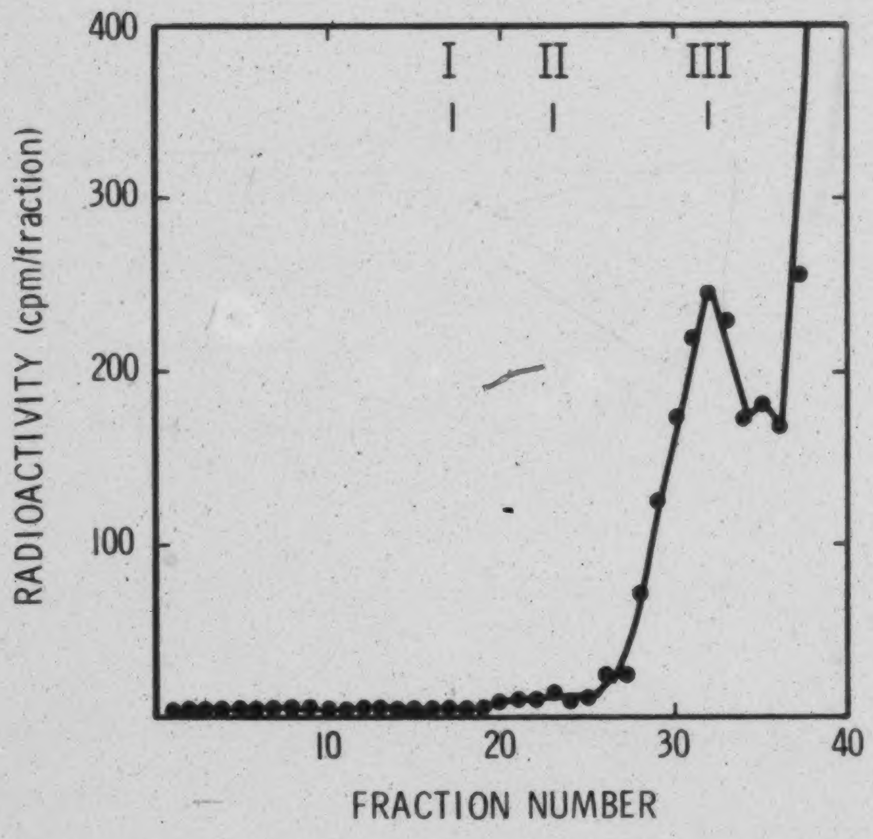

A
B
I
II
III
2000
1500
1000
500
800
600
400
200
4.0
3.0
2.0
1.0
10
20
30
40
RADIOACTIVITY (cpm / FRACTION)
ABSORBANCE (260 nm)
FRACTION NUMBER

equivalent to that noted for cytosol Peak III protein (Fig. 4.2B). On the other hand, nuclear extracts from the prostates of castrated rats contained only the larger form of receptor appearing in the void volume. Thus the steroid binding activity of receptor with a Stokes' radius of 24 Å, disappears within 24 hours after castration. Since cellulose phosphate Peak 2a is also lost after castration (Fig. 4.9), it is reasonable to believe that Sephadex Peak III receptors and cellulose phosphate Peak 2a receptors are equivalent. Similarly, cellulose phosphate Peak 1 receptor of the nucleus is probably the same binding protein as that recovered in the void volume after Sephadex G-200 gel filtration.

3. Repeat Chromatography of Nuclear Peak III

To investigate the possibility that the dihydrotestosterone binding activity recovered in the void volume after gel filtration through Sephadex G-200 arises as a result of aggregation of Peak III receptors, experiments were conducted in which isolated Peak III proteins were incubated and subjected to repeat gel filtration. Fractions at the center of Peak III (fractions 32 to 34 in Fig. 4.11A) were pooled and incubated with [1,2-^{3}H]dihydrotestosterone. A sample of 1 ml was applied to a Sephadex G-200 column and subsequently eluted from the column with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The results of this experiment, depicted in Fig. 4.11, indicate that there was no measurable change in the elution volume of nuclear Peak III radioactivity. Hence, it appears

Fig. 4.11. Repeat chromatography of Sephadex Peak III nuclear receptor on Sephadex G-200. Fractions 32 to 34 from the experiment shown in Fig. 4.10A were combined and incubated in the presence of 1×10^{-11} moles of [1,2-^{3}H]dihydrotestosterone at 25° C for 90 min. 1 ml of the sample (total volume of 3.9 ml) was then applied to a Sephadex G-200 column and eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The radioactivity in each fraction of 1.5 ml was measured.


RADIOACTIVITY (cpm/fraction)
FRACTION NUMBER
400
300
200
100
10
20
30
40
I
II
III

unlikely that the formation of the large receptor complex is due to aggregation of smaller receptor units during the experiment. However, this conclusion is tentative in view of the possibility that conditions used may not be adequate to produce the aggregation effect.

4. Effects of DNase, RNase and Pronase on Nuclear Receptors

It has been suggested that the 3-4S steroid-receptor complex recovered from target cell nuclei may itself become bound to another nuclear macromolecule. While the chemical composition of the latter complex was not elucidated, it was thought to be a protein which was only found in the nuclei of hormone responsive tissues (Tymoczko and Liao, 1971). More recently, however, King and Gordon (1972) demonstrated that the 4S nuclear receptor complex from rat uterus binds to DNA. Liao _et al_. (1973), on the other hand, have presented evidence implicating an RNA containing fraction as the principal nuclear entity that binds the androgen-receptor complex.

Because of the uncertainty in the evidence, experiments were performed in which labelled nuclear extracts were incubated with various digestive enzymes and chromatographed on Sephadex G-200. Nuclei were obtained from prostates of castrated rats and appropriate extracts were incubated with [1,2-^{3}H]dihydrotestosterone at 25° C for 30 min. At the end of this period, RNase or DNase was added, and the samples were incubated at 25° C for a further 60 min. The reaction mixtures were subjected to gel filtration on Sephadex G-200, and the eluted fractions were analyzed for radioactivity and absorbance at 260 nm. The results shown in Fig. 4.12A reveal that DNase caused a significant drop in the amount of absorbing material present in the void volume of the column (compare with Fig. 4.10B). However, there was no accompanying loss in the amount of $[1,2-^{3}H]$-dihydrotestosterone associated with this fraction. In the experiment with RNase, a slight decline was observed in both the height of the peak of radioactivity and in the absorbance spectrum (Fig. 4.12B). Since RNase did not cause a significant drop in the radioactivity-bound to prostatic nuclear receptors of intact rats (Table 3.3), the results presented here (Fig. 4.12B) are probably due to experimental variation. Thus under these conditions, neither DNase nor RNase produced a significant depression in the binding of radioactivity associated with Sephadex Peak I.

When pronase was incubated with nuclear extracts from prostates of intact rats, the results shown in Fig. 4.13 were obtained. The radioactivity that in control experiments (Fig. 4.10A) was bound to small receptors (nuclear Peak III), was not evident; however, an appreciable amount of radioactivity was recovered in the void volume. Apparently, the steroid binding sites in the nuclear Peak I fraction are less susceptible to proteolytic digestion than those in the Peak III area of the column.

Fig. 4.12. Effects of DNase and RNase on the binding of dihydrotestosterone. In each experiment, approximately 5×10^{7} nuclei from castrated rats were extracted with 0.6 M NaCl in Tris-EDTA buffer, pH 7.0. The nuclear extracts (1 to 2 ml) were incubated with 1×10^{-11} moles of $[1,2-^{3}H]$-dihydrotestosterone for 30 min at 25° C. At this time 500 μg of either DNase or RNase were added to the incubation mixture. After a further 60 min at 25° C, the samples were applied to a Sephadex G-200 column and eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The absorbance at 260 nm and radioactivity in each 1.5 ml fraction were measured. Panel A, treatment with DNase; radioactivity (●———●), absorbance (○———○). Panel B, treatment with RNase; radioactivity (●———●), absorbance (○———○).

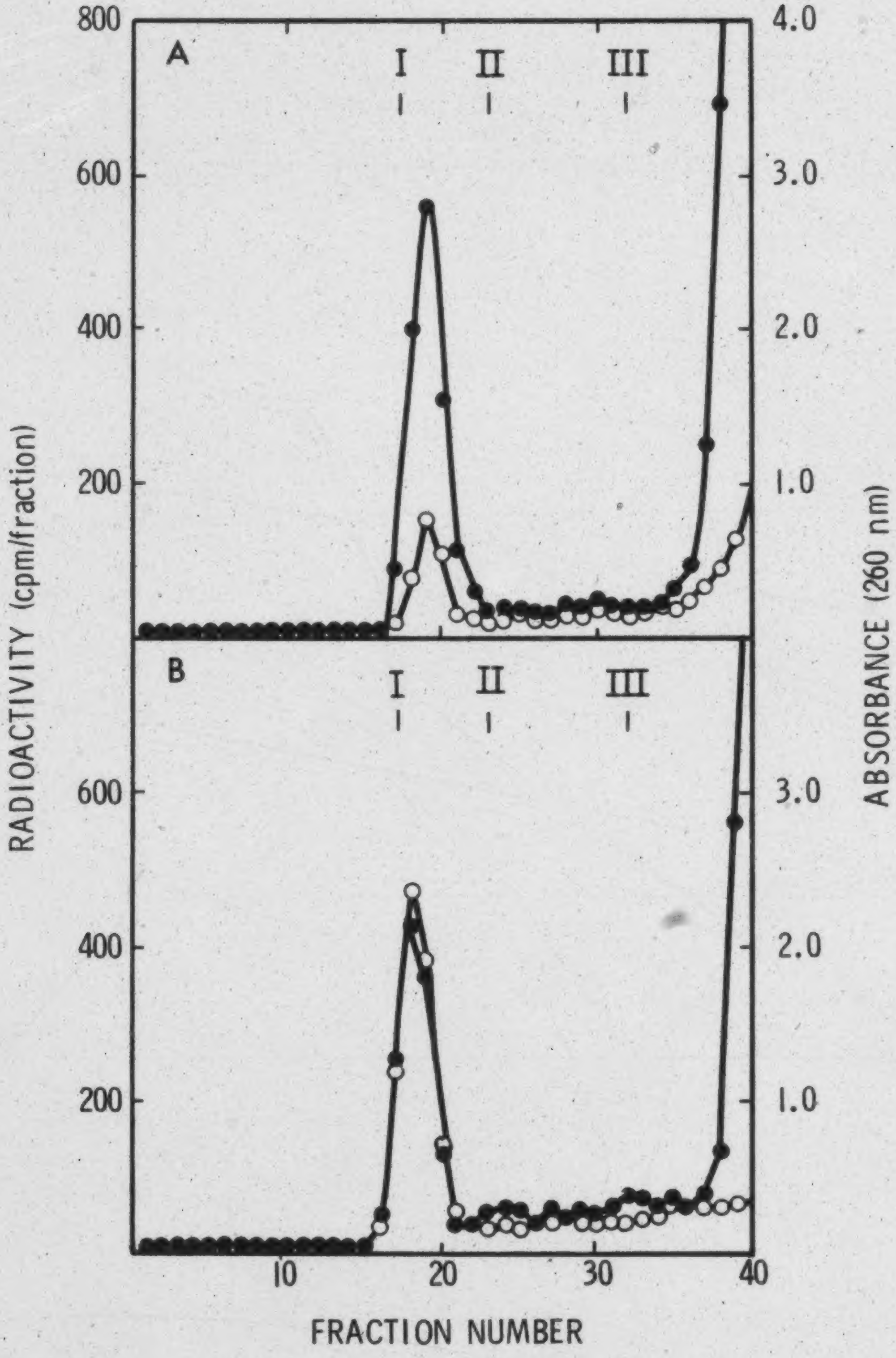
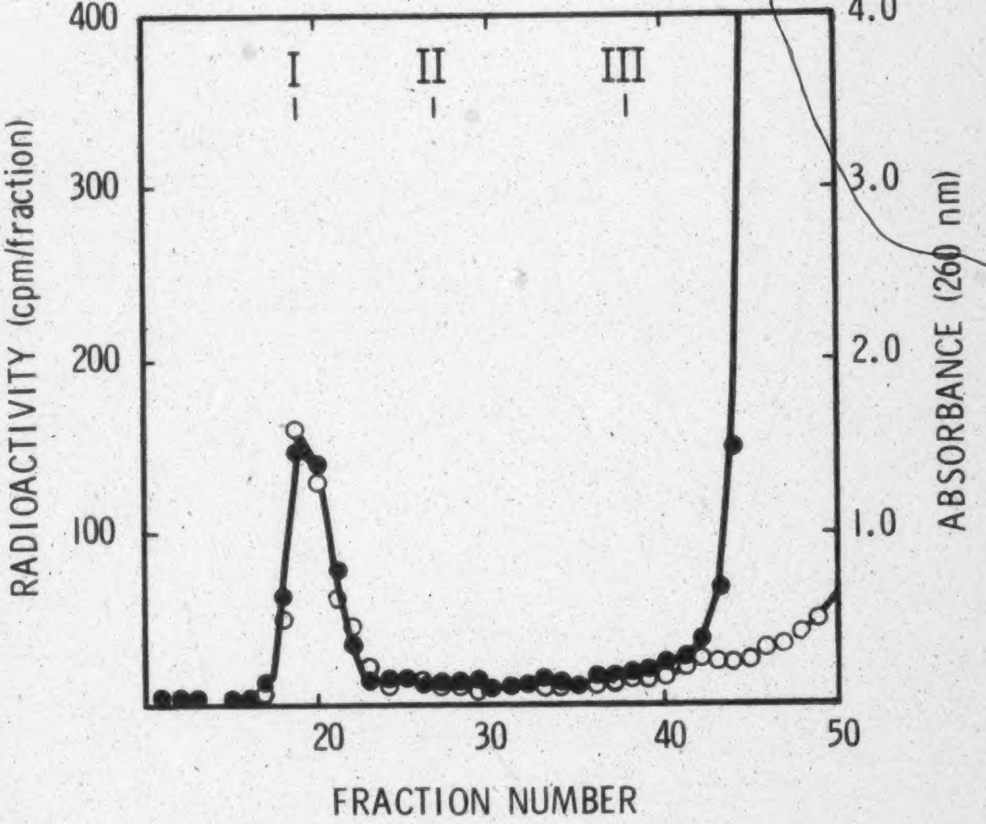

A
I
II
III
B
I
II
III
800
600
400
200
600
400
200
4.0
3.0
2.0
1.0
3.0
2.0
1.0
10
20
30
40
RADIOACTIVITY (cpm/fraction)
ABSORBANCE (260 nm)
FRACTION NUMBER

Fig. 4.13. Effect of pronase on the binding of dihydrotestosterone. A nuclear extract was prepared from 5 x 10^{7} nuclei obtained from intact animals. The sample (1 to 2 ml) was incubated with 1 x 10^{-11} moles of [1,2-^{3}H]dihydrotestosterone at 25° C for 30 min at which time 500 µg of pronase was added. After an additional 60 min incubation at 25° C the sample was applied to a Sephadex G-200 column and 1.3 ml fractions were eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. Radioactivity ●——● ; absorbance, ○——○ .

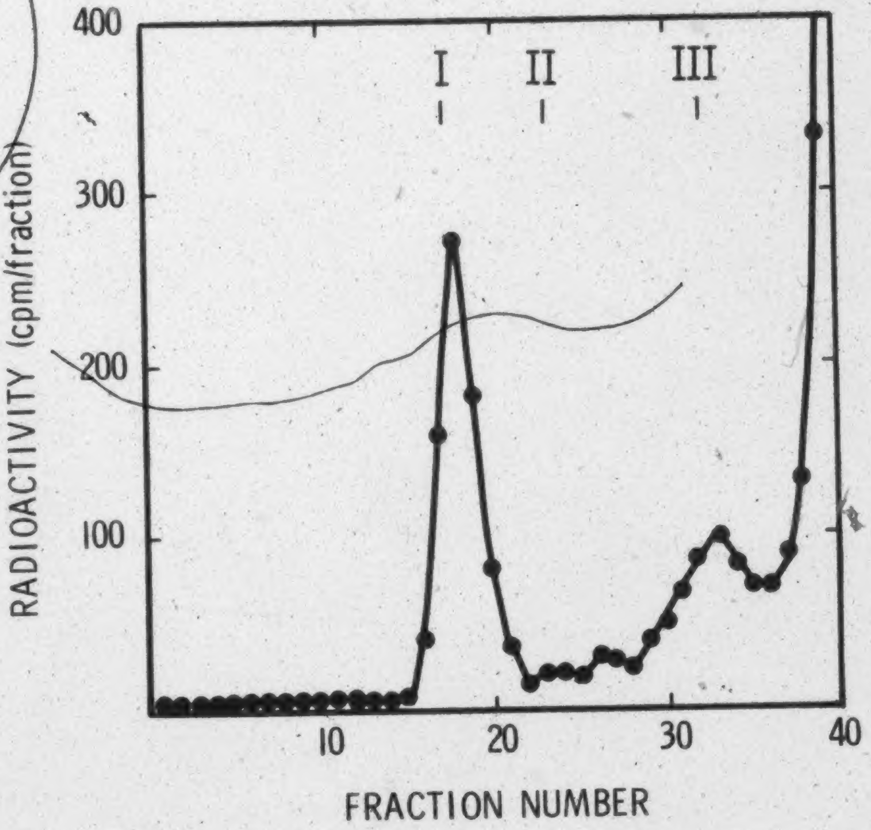

RADIOACTIVITY (cpm/fraction)
400
300
200
100
ABSORBANCE (260 nm)
4.0
3.0
2.0
1.0
I
II
III
20
30
40
50
FRACTION NUMBER

5. Testosterone Receptors in Nuclei

The binding studies described in Chapter III indicate that testosterone binds to nuclear receptors with a relatively high affinity (Ka $\sim 10^7$ M^{-1}). Therefore it was expected that binding of testosterone to specific peaks could be readily demonstrated. Gel filtration using Sephadex G-200 was the technique employed in this study since the previous results (Figs. 4.1 and 4.3) with cytosol receptors indicated that testosterone binding could best be demonstrated in this manner.

Nuclear extracts, prepared from prostates of intact rats, were incubated at 25° C for 90 min with [1,2-^{3}H]testosterone. Following this, the sample was applied to a Sephadex G-200 column and then eluted from the column with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The results shown in Fig. 4.14 indicate that the profile of radioactivity was qualitatively similar to that observed when [1,2-^{3}H]dihydrotestosterone was used as substrate (compare with Fig. 4.10A). The two radioactive peaks were almost coincident with Sephadex Peaks I and III. However, testosterone binding differed in two respects. First, the quantity of ^{3}H-steroid bound to nuclear receptors was considerably less than that observed in parallel studies using [1,2-^{3}H]dihydrotestosterone. Secondly, in the experiment with testosterone, the amount of radioactivity in Peak I was much greater than in Peak III; by contrast, the radioactivity in these peaks was almost the same in experiments with dihydrotestosterone.

Fig. 4.14. Binding of testosterone to intranuclear receptors. A nuclear extract (1 to 2 ml) corresponding to 5×10^{7} nuclei, isolated from intact rats, was incubated at 25° C for 90 min with 1×10^{-11} moles of [1,2-^{3}H]testosterone. On completion of incubation, the sample was analyzed on a Sephadex G-200 column (1 cm x 90 cm). Fractions of 1.5 ml were collected and measured for radioactivity.



I
II
III
400
300
200
100
10
20
30
40
RADIOACTIVITY (cpm/fraction)
FRACTION NUMBER

PART III - Serum Receptors

Although precautions were taken to ensure that contamination of the cytoplasmic and nuclear preparations by serum proteins was minimized, it remained possible that some of the dihydrotestosterone binding ascribed to intracellular proteins resulted from binding to serum proteins. Both steroid-binding globulin and serum albumin are capable of binding androgens (Westphal, 1971). Therefore experiments were conducted in which serum proteins were incubated with radioactive dihydrotestosterone and then examined by analysis on Sephadex G-200 and cellulose phosphate columns.

Samples of blood were collected in heparinized tubes from the jugular vein of castrated rats. Serum proteins were separated from erythrocytes by centrifugation at 500 x g for 10 min in a Sorvall GLC-1 centrifuge (HL-4 rotor, R_{avg} 12.5 cm). The upper layer was removed and then respun at 500 x g to further clarify the serum fraction. The final supernatant was decanted into clean tubes and aliquots were taken for protein determinations.

Samples containing 15 to 20 mg of serum protein were brought to a final volume of 1 ml with Tris-EDTA buffer, pH 7.0, and then incubated at 25° C for 90 min in the presence of [1,2-^{3}H]-dihydrotestosterone. On completion of incubation, the proteins were either applied directly to a Sephadex G-200 column or passed through a Sephadex G-25 column and then applied to a cellulose phosphate column.

The results of these experiments are shown in Figs. 4.15 and 4.16 respectively. Two peaks of radioactivity were observed in the Sephadex G-200 chromatogram. The first peak eluted from the column was relatively small and diffuse, and had an elution volume consistent with that of steroid-binding globulin. The second peak, which contained the majority of radioactivity, was recovered in an elution volume that corresponded to a globular protein with a molecular weight of approximately 64,000 - 68,000. The latter protein is probably albumin. Both types of steroid binding measured in these experiments had different elution volumes than those observed in similar experiments with intracellular receptors of the rat prostate (compare with Fig. 4.3B).

When the serum proteins were analyzed on cellulose phosphate two peaks of radioactivity were again observed (Fig. 4.16). Although, it was not established which peak corresponded to albumin and which to steroid-binding globulin, the positions of the peaks in the cellulose phosphate chromatogram differed from cellulose phosphate Peaks 1, 2, and 2a (compare with Figs. 4.1 and 4.8). Clearly, both Sephadex G-200 and cellulose phosphate chromatography allow one to differentiate between the androgen binding proteins of the serum and of the prostate. Since neither steroid-binding globulin nor serum albumin binding activity is detected in prostatic preparations, contamination by extracellular proteins is probably minimal.

Fig. 4.15. Demonstration of in vitro labelled steroid-receptors of serum on Sephadex G-200. Approximately 18 mg of serum protein in 1 ml of Tris-EDTA buffer, pH 7.0 was incubated at 25° C for 90 min with 1 x 10^{-11} moles of [1,2-^{3}H]dihydrotestosterone. Following incubation, the sample was applied to a Sephadex G-200 column (1 cm x 90 cm) and eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. Fractions of 1.5 ml were collected and radioactivity was measured.

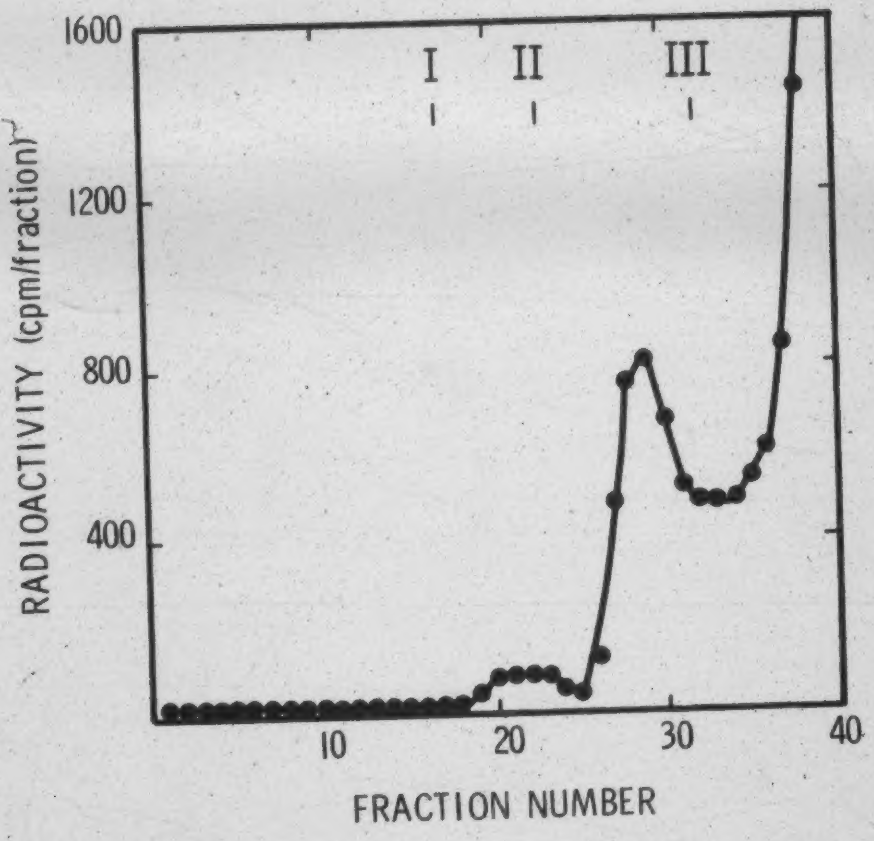
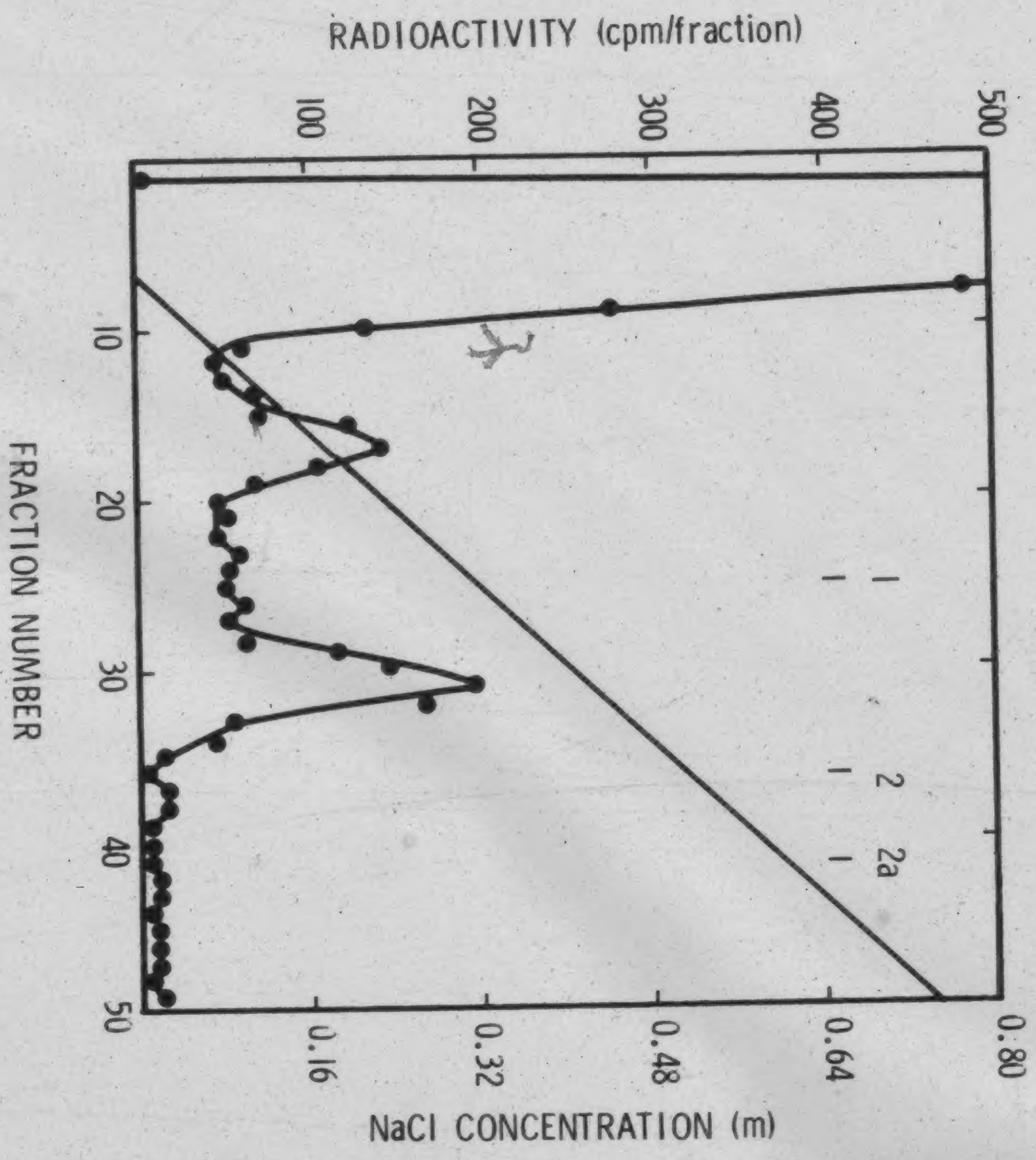

1600
1200
800
400
RADIOACTIVITY (cpm/fraction)
I
II
III
10
20
30
40
FRACTION NUMBER

Fig. 4.16. Demonstration of in vitro labelled steroid-receptors of serum on cellulose phosphate. Approximately 20 mg of serum protein was incubated as described in the legend of Fig. 4.16. Following incubation the sample was passed through a Sephadex G-25 column. The void volume was then analyzed on cellulose phosphate under the same conditions as described in the legend to Fig. 4.1.


RADIOACTIVITY (cpm/fraction)
100
200
300
400
500
2
2a
FRACTION NUMBER
10
20
30
40
50
0.16
0.32
0.48
0.64
0.80
NaCl CONCENTRATION (m)

D. Discussion

In this investigation four types of receptor molecules for dihydrotestosterone were observed in prostatic cytosol. Examination of *in vitro* labelled cytosol extracts on cellulose phosphate columns revealed the presence of two discrete peaks of radioactivity (Peaks 1 and 2) (Fig. 4.1). Further characterization of these peaks on Sephadex G-200 demonstrated that several forms of dihydrotestosterone binding proteins were present in prostatic cytosol. Peak 1, obtained with cellulose phosphate, was found to contain receptor proteins with a Stokes' radius of about 48 Å (Fig. 4.4A), whereas receptor proteins of at least three different molecular sizes were detected in Peak 2 (Fig. 4.2B). These had Stokes' radii of 24 Å, 48 Å, and >48 Å. In this investigation, it was also found that certain of the cytosol receptor complexes undergo a molecular size reduction in the presence of high molar salt. For example, when *in vitro* labelled cytosol extracts were run on Sephadex G-200 the distribution of radioactivity between Peak I and Peak II could be altered by changing the concentration of NaCl in the eluant. At high ionic strength (0.6 *M* NaCl) more radioactivity was bound to Sephadex Peak II; conversely, in the absence of NaCl, more radioactivity was bound to Sephadex Peak I (Fig. 4.2).

Study of the intranuclear binding of dihydrotestosterone furnished evidence of the existence of two receptors for dihydrotestosterone. Using cellulose phosphate, one nuclear receptor was eluted in a position corresponding to cellulose phosphate Peak 1 of cytosol (Fig. 4.8). The other nuclear receptor was eluted in a position close to, but not coincident, with cellulose phosphate Peak 2 of cytosol and was designated Peak 2a (Fig. 4.8). When Sephadex G-200 was used to analyse the intranuclear binding of dihydrotestosterone, two peaks were again obtained, one corresponding to Sephadex Peak I of cytosol and the other corresponding to Sephadex Peak III of cytosol. Castration caused a marked reduction in the size of cellulose phosphate Peak 2a and Sephadex Peak III of the nucleus. These parallel changes suggest that cellulose phosphate Peak 2a and Sephadex Peak III represent the same receptor. The relative stability of cellulose phosphate Peak 1 and Sephadex Peak I suggests that these peaks represent a second intranuclear binding site.

Comparison of the chromatographic properties of the cytoplasmic and nuclear receptors as determined by *in vitro* experiments, indicates that there is a single receptor that is common to both the cytoplasm and nucleus. This receptor has a Stokes' radius of 24 Å and both the cytoplasmic and nuclear forms have similar, though not identical, affinity for cellulose phosphate (compare Peaks 2 and 2a in Figs. 4.1 and 4.8).

The 24 Å nuclear receptor is a protein as established by its sensitivity to pronase. The chemical nature of the presumed second nuclear receptor is less clear. Binding of radioactivity to Sephadex Peak I (castrated rats) is not completely abolished by pronase (Fig. 4.13). It is not sensitive to DNase digestion and only slightly sensitive to RNase digestion (Fig. 4.12). The reason for the relative resistance of this peak to these treatments is not clear. It is possible that the binding to Sephadex Peak I represents a different type of complex than the binding to Sephadex Peak III. This view is consistent with the suggestion of Tymocyzko and Liao (1971) that the nucleus contains an acceptor site which binds steroid or steroid-protein complex transported across the nuclear membrane.

No binding of [1,2-^{3}H]testosterone to cytosol protein could be demonstrated on cellulose phosphate (Fig. 4.1). The isolation of testosterone-protein complexes was more successful when Sephadex G-200 was used and two peaks of radioactivity were obtained (Fig. 4.3). The first peak was eluted in the void volume and the second peak was eluted in a position similar to Peak III. Both peaks were smaller than those obtained in comparable experiments with [1,2-^{3}H]dihydrotestosterone. Also, the presence of 0.6 M NaCl in the elution buffer did not give rise to a testosterone binding peak in the cytosol corresponding to Sephadex Peak II (Fig. 4.3). Together this evidence suggests that the receptor sites for testosterone are different from those for dihydrotestosterone;

however, the results may also be attributable to the possibility that experimental conditions used were inadequate to obtain maximum binding of testosterone. In the next Chapter, experiments are described which were undertaken to establish whether these *in vitro* results could be related to the *in vivo* action of androgens.

CHAPTER V

IN VIVO BINDING STUDIES

A. Introduction

There is considerable evidence to suggest that under *in vitro* conditions cytosol steroid-receptor in the presence of dihydrotestosterone stimulates the incorporation and retention of dihydrotestosterone by isolated nuclei (Fang *et al*., 1969; Fang and Liao, 1971; Steggles *et al*., 1971). While such an observation would be expected if steroid-receptors migrate from cytoplasm to nucleus, it is not certain whether this migration occurs *in vivo*. Generally speaking, *in vivo* studies of androgen uptake and retention by rat ventral prostate have not been concerned with the effect of binding proteins in regulating the intracellular distribution of androgens. Accordingly, experiments described in this chapter were undertaken, first, to differentiate between cytoplasmic and nuclear steroid-receptors under *in vivo* conditions, secondly, to compare these with steroid-receptors defined under *in vitro* conditions, and thirdly, to assess the function of steroid-receptors in terms of their role in the transport of steroid from cytoplasm to the nucleus.

B. Materials and Methods

1. Preparation of Cytosol and Nuclear Fractions

Approximately 24 hours after castration, male rats were eviscerated, functionally hepatectomized, and then injected intravenously with 150 µCi of radioactive steroid. (It has been shown that ablation of viscera causes significant increase in the amount of radioactive testosterone taken up by the rat ventral prostate (Bruchovsky and Wilson, 1968a)). After 10, 30, 60 or 120 min the rats were killed by decapitation and their prostates were removed.

The procedures used for the homogenization of prostatic tissue and isolation of nuclei and cytosol were identical to those used in *in vitro* experiments. Aliquots were taken for the measurement of radioactivity, for the counting of nuclei, and in some instances, for the identification of steroids.

2. Column Chromatography Procedures

To quantitate steroid-protein binding *in vivo*, suitable extracts from cytosol or nuclei were chromatographed on Sephadex G-25 columns (1 cm x 40 cm) as before. Fractions containing the steroid-receptor complex were analyzed for radioactivity and protein. In some experiments the *in vivo* labelled extracts were run on cellulose phosphate or Sephadex G-200 columns. The chromatographic conditions were identical to those employed in the *in vitro* experiments. When necessary, steroid metabolites were recovered from appropriate fractions and identified by thin-layer chromatography.

C. Results

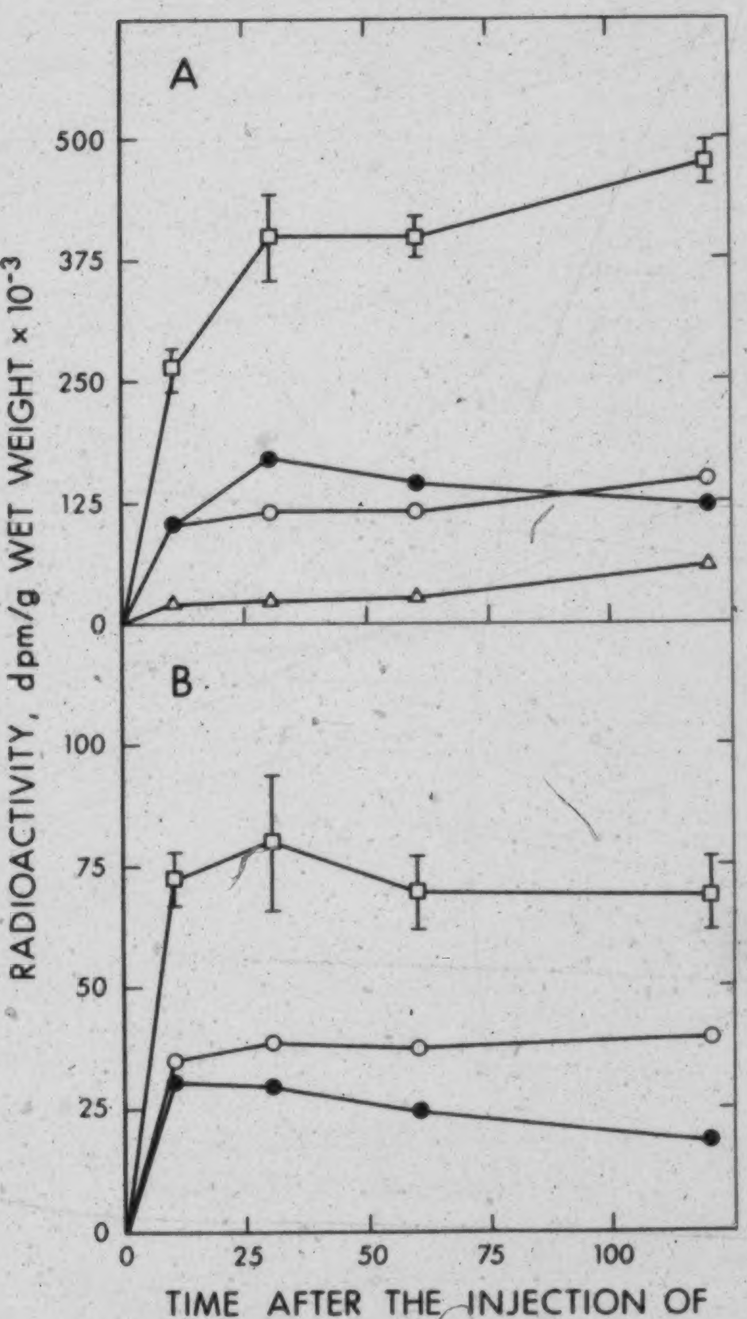
PART I - In Vivo Cytosol Receptors

1. Incorporation of ^{3}H-Androgens into Cytosol after the Pulse Injection of [1,2-^{3}H]Testosterone

In order to obtain insight into the relationship between the appearance of androgens in prostatic cytosol and the binding of androgens to cytosol receptor, the ^{3}H-steroids associated with these two fractions were quantitated and identified at 10, 30, 60 and 120 min after the intravenous administration of 150 μCi (1 μg) of [1,2-^{3}H]testosterone to castrated, functionally hepatectomized rats. The radioactivity in cytosol is plotted as a function of time in Fig. 5.1A (upper curve) and it can be seen that the amount of label rises sharply to 400 x 10^3 dpm/g wet weight at 30 min and little further change occurs between 30 min and 120 min. The lower curves in Fig. 5.1A indicate the relative amounts of testosterone, dihydrotestosterone and androstanediol present in this fraction. Testosterone and dihydrotestosterone are clearly the dominant metabolites at all times and are recovered in nearly equal proportions.

The results of parallel measurements on the labelling of cytosol receptor are shown in Fig. 5.1B. Labelling of the receptor was virtually complete by 10 min and there was little change from the level of 75 x 10^3 dpm reached at this time. The amount of

Fig. 5.1. Incorporation of ^{3}H-androgens into cytosol after the pulse injection of [1,2-^{3}H]testosterone. Groups of 3 to 5 rats castrated 24 hours previously were functionally hepatectomized and injected with 150 μCi of [1,2-^{3}H]testosterone (sp. act. 5 mCi/.032 mg). Then 10, 30, 60 and 120 min later the rats were killed and samples of prostate were fractionated as described in Chapter II; appropriate aliquots were assayed for radioactivity and steroid constituents were identified by thin-layer chromatography. Protein was precipitated with ammonium sulphate (80% saturation) and applied to a column (1 cm x 40 cm) of Sephadex G-25. The column was eluted with Tris-EDTA buffer, pH 7.0 and the void volume was collected. An aliquot was removed for measurement of radioactivity and the remainder of the fraction was extracted with chloroform-methanol (2:1, v/v); neutral metabolites were identified by thin-layer chromatography. Each value represents the mean of at least 3 separate experiments; the standard error of the mean is shown for measurements of radioactivity. Panel A, radioactivity and androgens recovered in cytosol; panel B, in cytosol receptor. Total radioactivity (mean ± S.E.) □———□ ; dihydrotestosterone, ○———○ ; testosterone, ●———● ; androstanediol, △———△ .


A
B
RADIOACTIVITY, dpm/g WET WEIGHT × 10^{-3}
500
375
250
125
0
100
75
50
25
0
0
25
50
75
100
TIME AFTER THE INJECTION OF
[1,2-^{3}H] TESTOSTERONE (min)

dihydrotestosterone bound remained stable at 35×10^3 dpm; the amount of testosterone declined slightly from 30×10^3 dpm at 10 min to 20×10^3 dpm at 120 min. However, for the most part, the relative amounts were only slightly different from those in the cytosol fraction. Only a trace amount of androstanediol (less than 2%) was recovered from the steroid-receptor complex despite the fact that this steroid represents 7 to 14% of the radioactivity in cytosol. This observation is in keeping with the view that the selectivity of binding, and by inference, of transport, is established at the level of the cytosol receptor.

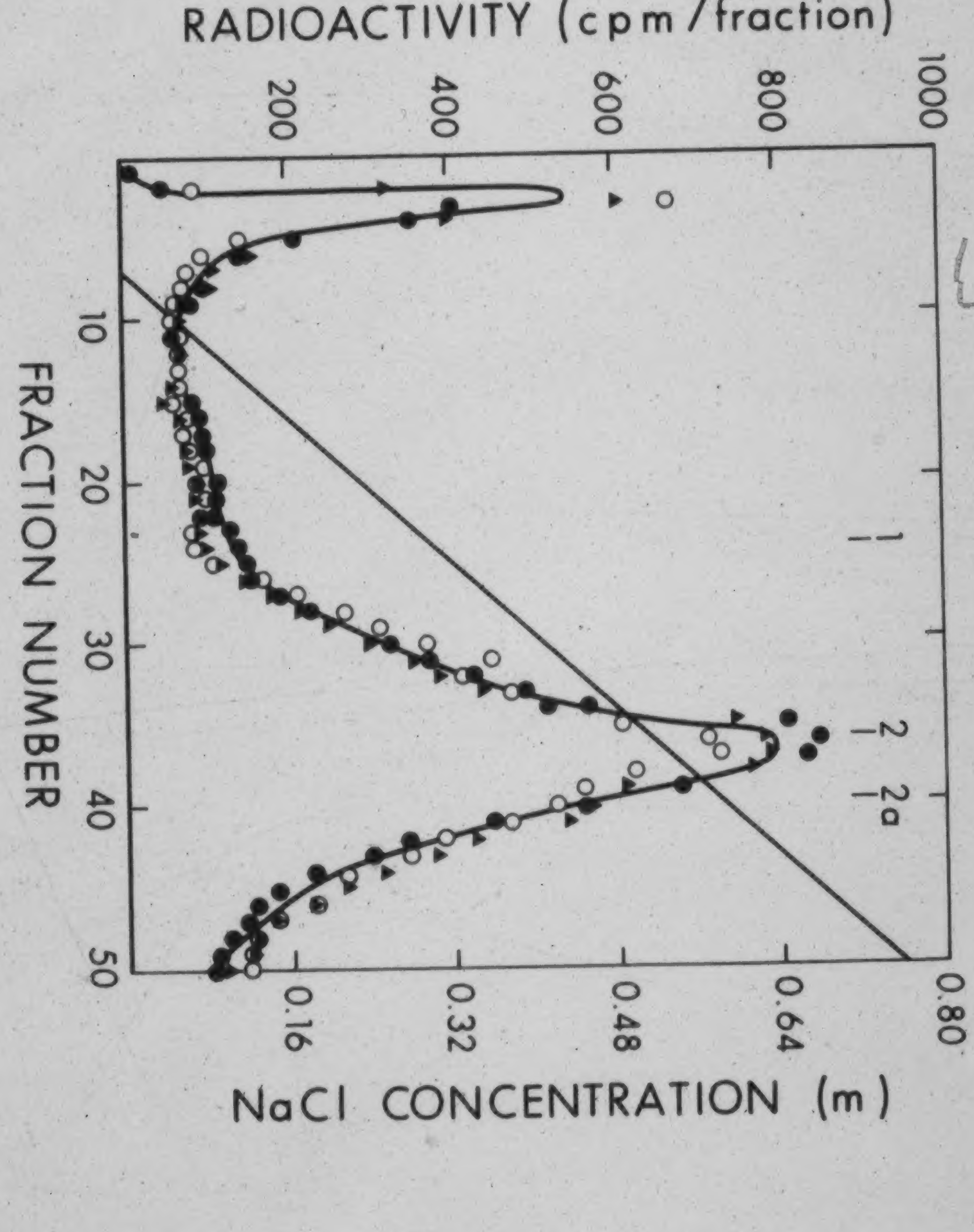
2. Partial Purification of Cytosol Receptors using Cellulose Phosphate

Experiments were carried out to characterize the steroid-receptors in cytosol using cellulose phosphate. At 10, 30 and 60 min following the administration of radioactive testosterone to 24-hour castrated rats, prostatic cytosol was isolated and treated with ammonium sulphate at 80% saturation. The precipitates were desalted by gel filtration, applied to a cellulose phosphate column, and eluted with a salt gradient. As shown in Fig. 5.2 virtually all of the labelled receptor was recovered in the Peak 2 area. Moreover, the amount of radioactivity in this peak was relatively constant after 10, 30 and 60 min of *in vivo* incubation, and no radioactivity corresponding to Peak 1 was detected at any time.

Fig. 5.2. Demonstration of *in vivo* cytosol receptors on cellulose phosphate. Groups of 4 to 5 male rats castrated 24 hours previously were functionally hepatectomized immediately prior to receiving intravenous injections of 150 µCi [1,2-^{3}H]-testosterone. Then 10, 30 and 60 min later the rats were killed and cytosol was isolated from 1 g of the combined prostatic tissue of each group. Protein was precipitated with ammonium sulphate (80% saturation), desalted by gel-filtration on Sephadex gel and applied to a 1 cm x 15 cm column of cellulose phosphate. Receptor complex was eluted with an ionic gradient consisting of Tris-EDTA buffer, pH 7.0 containing 0 to 0.8 M NaCl. Fractions of 4.2 ml each were collected and the radioactivity in each fraction was measured. Radioactivity recovered;

●——● 10 min; ○——○ 30 min;

▲——▲ 60 min.

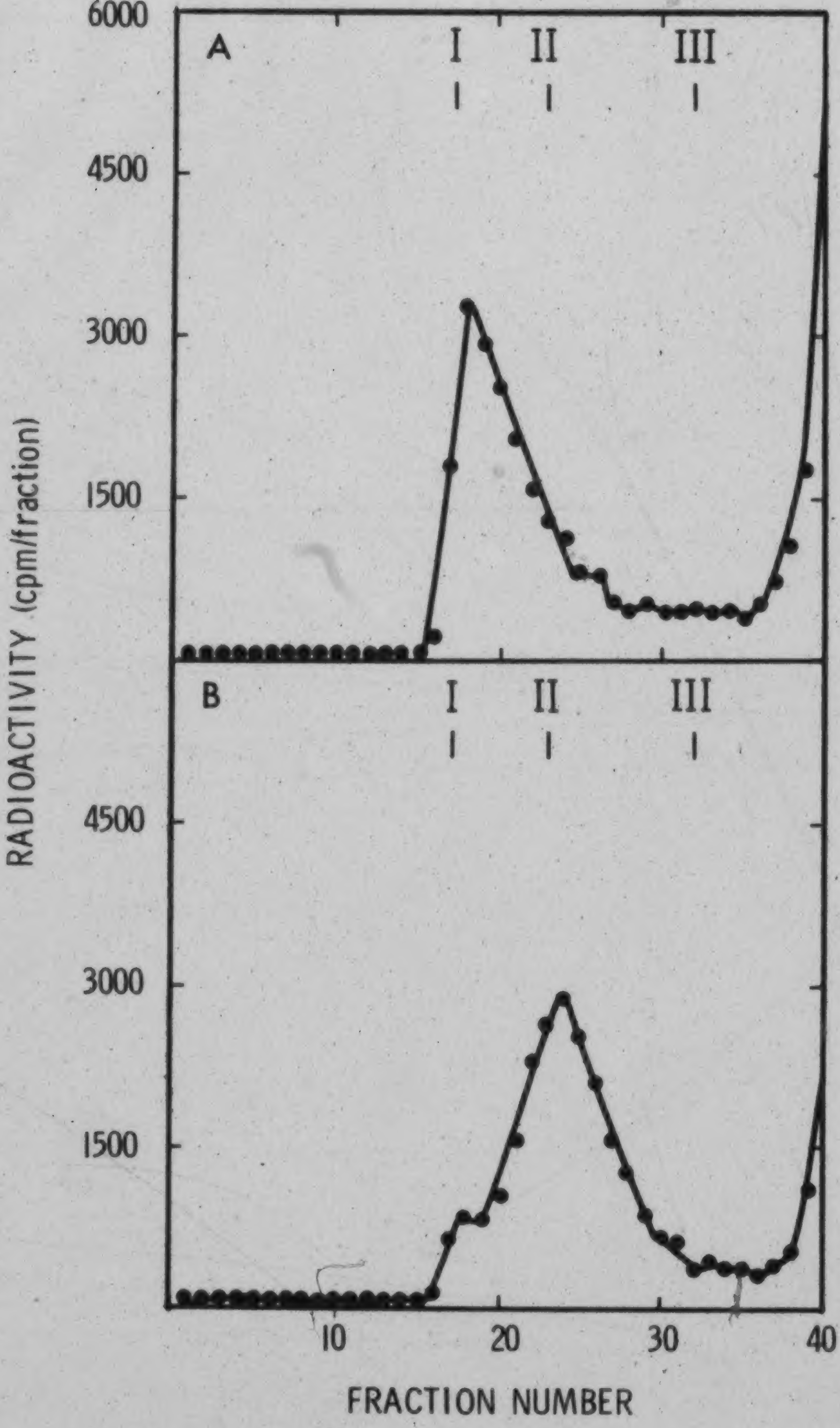

RADIOACTIVITY (cpm/fraction)
200
400
600
800
1000
FRACTION NUMBER
10
20
30
40
50
NaCl CONCENTRATION (m)
0.16
0.32
0.48
0.64
0.80
1
2
2a

The ratio of ^{3}H-dihydrotestosterone to ^{3}H-testosterone in Peak 2 was found to be similar to that shown in Fig. 5.1. Thus it is clear that while *in vitro* cytosol binding occurs to both Peak I and Peak 2 proteins (Fig. 4.1), the *in vivo* binding occurs exclusively to Peak 2 protein. These observations suggest that the proteins which bind androgens exist in different forms depending on the experimental conditions.

3. Chromatography of Cytosol Steroid-Receptors on Sephadex G-200

Cytosol protein was chromatographed on Sephadex G-200 to further characterize steroid-receptors in cytoplasm. Cytosol extracts were obtained from the prostates of castrated rats injected with 150 μCi of [1,2-^{3}H]testosterone. The cytosol proteins were precipitated with ammonium sulphate, resuspended in buffer and chromatographed on Sephadex G-200. Fig. 5.3 shows the results of experiments in which rats were killed 60 min after the injection of radioactive testosterone. In the absence of NaCl only Peak I radioactivity is recovered in association with cytosol proteins (Fig. 5.3A). In the presence of 0.6 M NaCl (Fig. 5.3B) radioactivity is recovered in Peak II and, to a lesser extent in Peak I. Thus the effect of NaCl *in vivo* is similar to its effect *in vitro* in causing an apparent transition of receptors from a larger to a smaller configuration. However, it is to be noted that Peak III which was prominent in *in vitro* experiments is conspicuously absent in these

Fig. 5.3. Demonstration of in vivo cytosol receptors on Sephadex G-200. Groups of 4 to 5 rats castrated 24 hours previously were functionally hepatectomized and then each animal was injected with 150 μCi of [1,2-^{3}H]testosterone. 60 min later the rats were killed and cytosol was isolated from 1 g of combined prostatic tissue of each group. Protein was precipitated with ammonium sulphate (80% saturation), resuspended in 1 ml of Tris-EDTA buffer, pH 7.0, and analyzed by gel filtration. Fractions of 1.5 ml were collected and the radioactivity in each was determined. Panel A, radioactivity recovered after elution of Sephadex G-200 column (1 cm x 90 cm) with Tris-EDTA buffer, pH 7.0. Panel B, radioactivity recovered when the column was eluted with buffer containing 0.6 M NaCl.

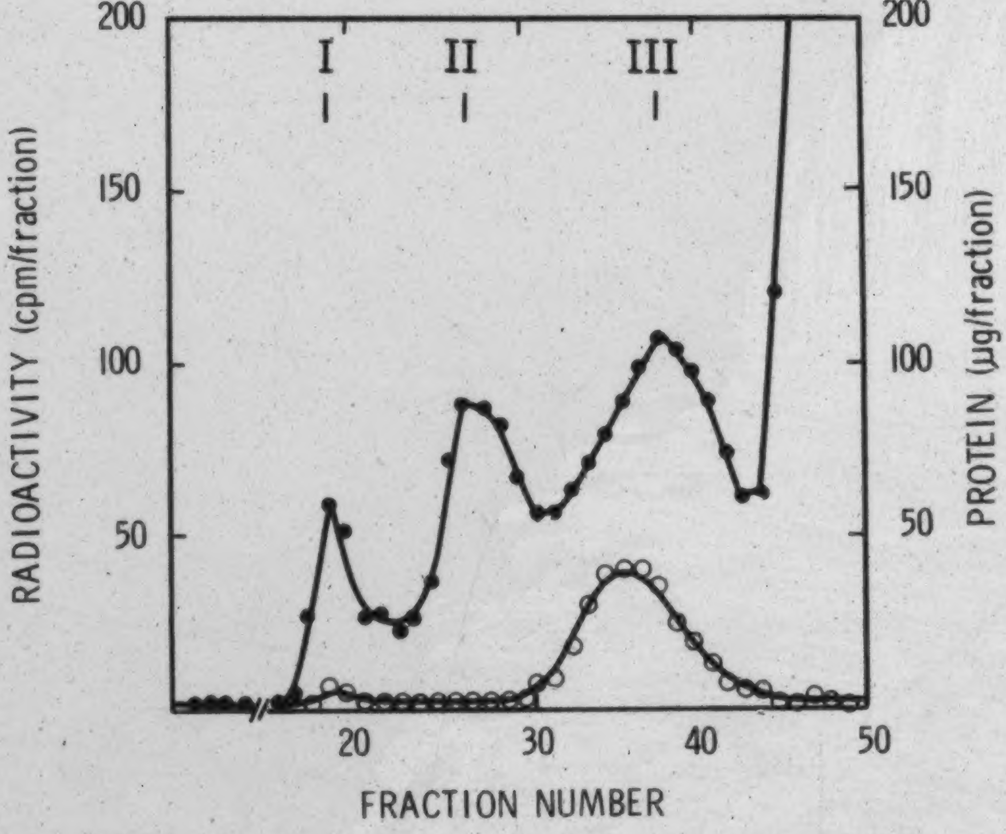

A
B
I
II
III
6000
4500
3000
1500
RADIOACTIVITY (cpm/fraction)
10
20
30
40
FRACTION NUMBER

in vivo experiments. Thus under *in vivo* conditions both cellulose phosphate Peak 1 and Sephadex Peak III are not detected.

4. Chromatography of Cellulose Phosphate Peak 2 Receptors on Sephadex G-200

The absence of Sephadex Peak III under *in vivo* conditions raises the question as to the significance of this peak as demonstrated under *in vitro* conditions. Since it was shown in Chapter IV that cellulose phosphate Peak 2 (labelled *in vitro*) could be rechromatographed on Sephadex G-200 to give rise to Sephadex Peaks I, II, and III it was of interest to determine whether cellulose phosphate Peak 2 (labelled *in vivo*) would give rise to a Sephadex Peak III. Accordingly, *in vivo* labelled cytosol protein, isolated after a 60 min pulse of [1,2-^{3}H]testosterone (150 μCi), was precipitated with ammonium sulphate, desalted, and applied to a cellulose phosphate column as before. After elution with an ionic gradient the Peak 2 area was pooled (Fig. 5.2, fractions 35 to 37) and incubated for 90 min at 25° C with [1,2-^{3}H]dihydrotestosterone. Following the *in vitro* incubation, the protein was precipitated with ammonium sulphate and then applied to a Sephadex G-200 column. The distribution of radioactivity the eluate is shown in Fig. 5.4A. It can be seen that the profile of radioactivity resembles that obtained after rechromatography of the *in vitro* labelled Peak 2 (Fig. 4.7B) but differs significantly from that obtained when protein from *in vivo* labelled cytosol is directly chromatographed on Sephadex G-200 (Fig. 5.3).

Fig. 5.4. Chromatography of cellulose phosphate Peak 2 on Sephadex G-200. Fractions corresponding to tubes 35 to 37 of Fig. 5.2 (after 60 min interval) were pooled. These fractions were reincubated at 25° C for 90 min with 2×10^{-11} moles of [1,2-^{3}H]dihydrotestosterone and then precipitated with ammonium sulphate (80% saturation). The sample was applied to a Sephadex G-200 column (1 cm x 90 cm) and eluted in 1.3 ml fractions with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. Radioactivity ●——● ; protein ○——○ .


200
150
100
50
I
II
III
RADIOACTIVITY (cpm/fraction)
PROTEIN (µg/fraction)
20
30
40
50
FRACTION NUMBER

These results can be summarized as follows. When prostatic cells are labelled *in vivo*, it is possible to recover receptors in cytosol that chromatograph in the position of Peak 2 on cellulose phosphate and in the positions of Peak I and Peak II on Sephadex G-200. It is clear that a transition is induced between Peak I and Peak II by altering the ionic strength of eluting buffer. At low ionic strength the formation of Peak I is favored and conversely at high ionic strength the formation of Peak II is favored.

Rechromatography of cellulose phosphate Peak 2 on Sephadex G-200 yields a third peak (Peak III) that is evident whenever *in vitro* incubations are used to label cytosol receptors. This conclusion is supported both by the data presented in Fig. 5.4 and that presented in Figs. 4.7B.

PART II - *In Vivo* Nuclear Receptors

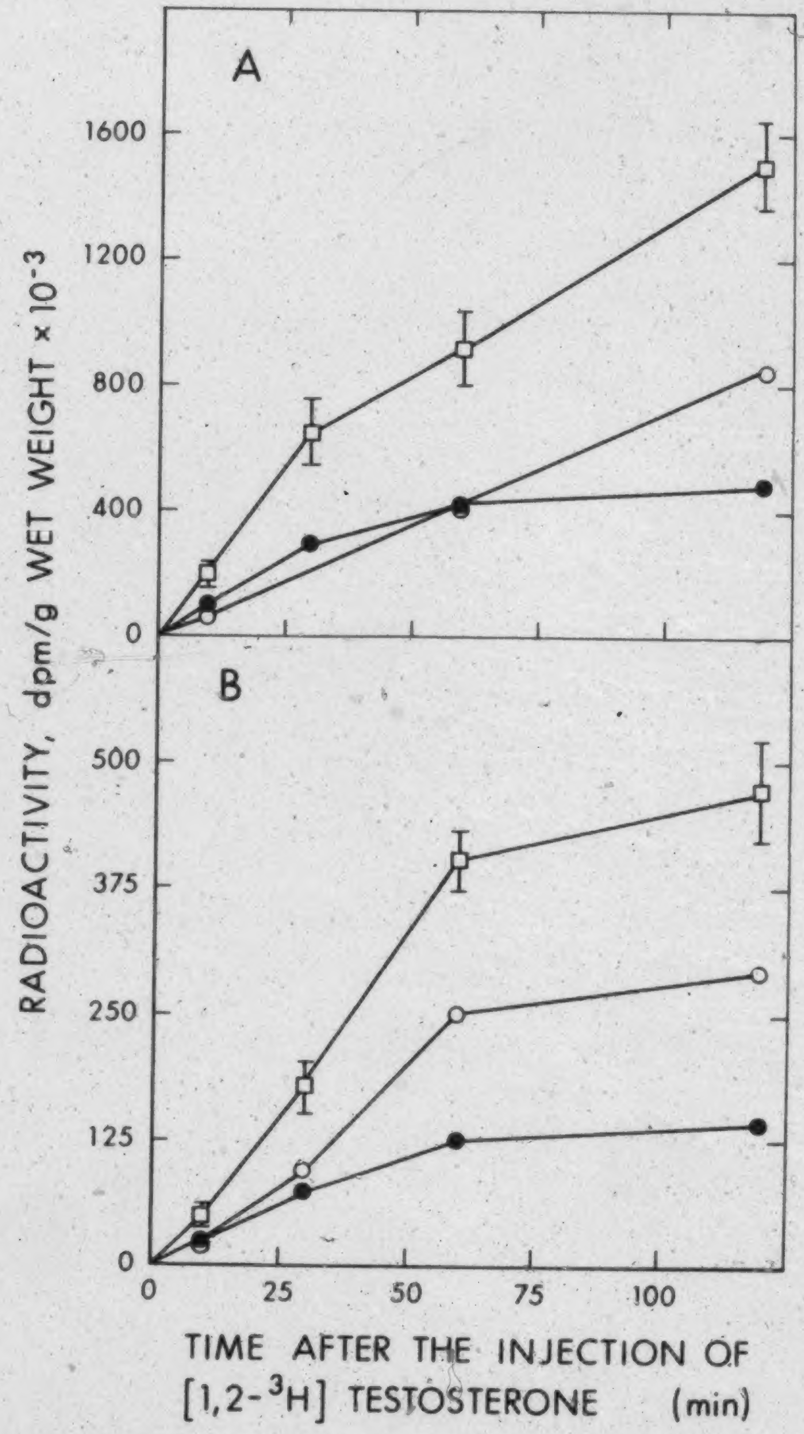
1. Incorporation of ^{3}H-Androgens into Nuclei after the Pulse Injection of [1,2-^{3}H]Testosterone

Experiments were performed to define any correlation between the accumulation of androgens in prostatic nuclei and the appearance of a specific steroid-protein complex in this fraction. Following surgery, rats were injected intravenously with 150 μCi (1 μg) of [1,2-^{3}H]testosterone and at suitable intervals were killed by decapitation. Nuclei were isolated from prostatic tissue and examined for content of radioactivity and of steroid-binding protein.

The incorporation of radioactivity by nuclei is plotted as a function of time in Fig. 5.5A (upper curve). The rate of uptake of ^{3}H-androgens was greatest during the initial 30 min; afterwards, incorporation continued to increase in a linear fashion until 120 min but at a lower rate. Between 10 and 120 min the amount of dihydrotestosterone increased uniformly; by contrast, the amount of testosterone reached a maximum level of approximately 400 x 10^3 dpm at 60 min. Whether this implies that the nuclei are saturated or that the supply of testosterone from the cytoplasm is attenuated is not clear. As might be predicted from the failure of androstanediol to bind to cytosol receptor, this compound is not recovered from nuclei.

The accumulation of ^{3}H-steroid-receptor in the nucleus is plotted as a function of time in Fig. 5.5B (upper curve). Radioactivity associated with this fraction increased linearly from 50 x 10^3 dpm at 10 min to 400 x 10^3 dpm at 60 min. Between 60 and 120 min there is a marked reduction in the rate of labelling such that the level attained at 120 min, 475 x 10^3 dpm, represents only a 20% increase over the level at 60 min. The percentage change is considerably lower than the 70% increase observed in the total nuclear level of radioactivity during the same interval. The steroid constituents of the steroid protein complex, as shown in Fig. 5.5B (lower curves), are dihydrotestosterone and testosterone. With increasing time the ratio of these two compounds changes from 1:1 at 10 min to 2:1 in favour of dihydrotestosterone at 120 min.

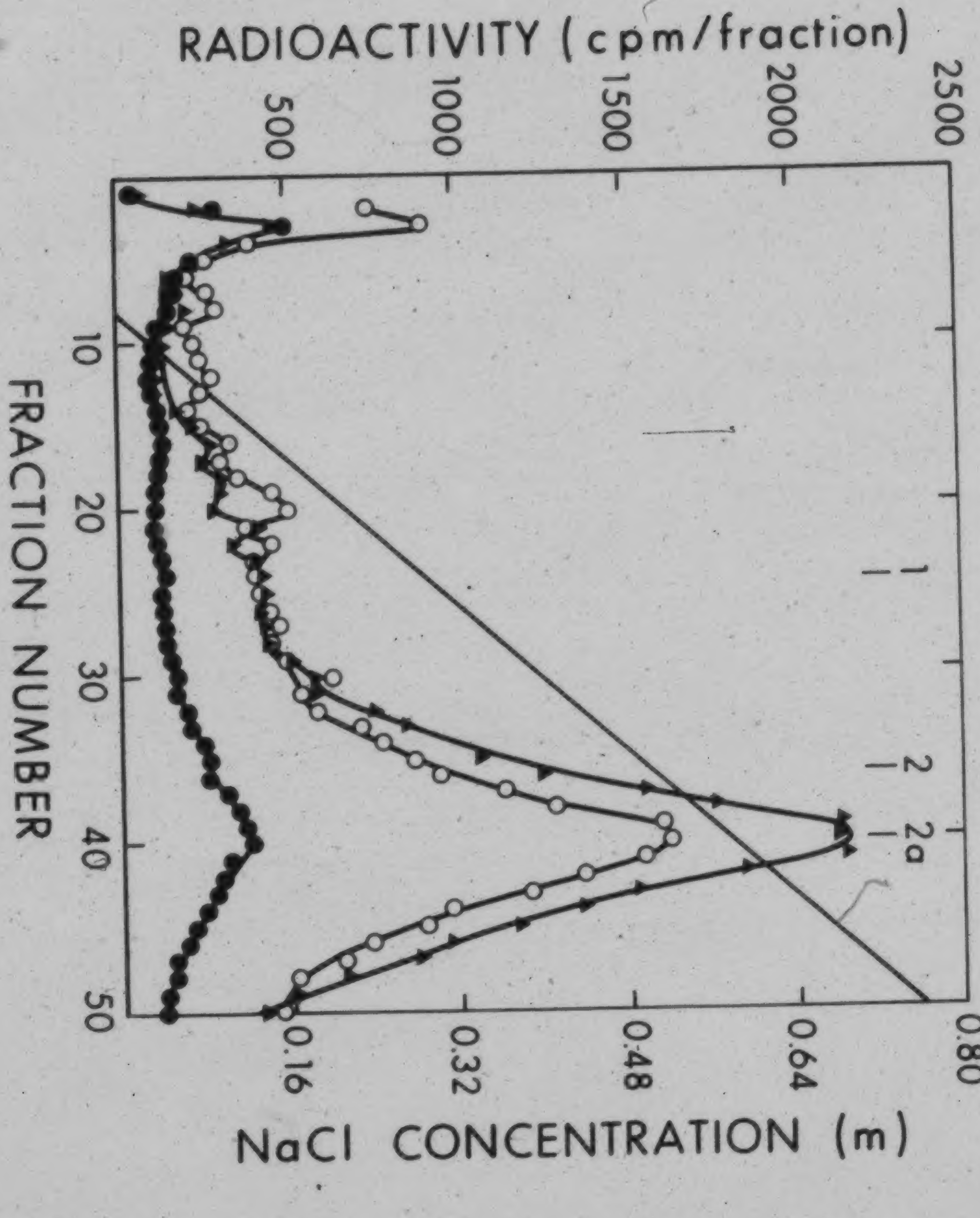
Fig. 5.5. Incorporation of ^{3}H-androgens by nuclei after the pulse injection of [1,2-^{3}H]testosterone. The experimental procedure is described in the text. Purified nuclei were obtained as described in Chapter II and were extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The amount of binding was determined by gel-filtration on Sephadex G-25. Samples of isolated nuclei and of nuclear receptor were assayed for radioactivity and the metabolites associated with each fraction was identified. Panel A, radioactivity and metabolites recovered in nuclei; panel B, in nuclear receptor. Total radioactivity (mean ± S.E.) □——□ ; dihydrotestosterone ○——○ ; testosterone ●——● .


A
B
RADIOACTIVITY, dpm/g WET WEIGHT × 10-3
1600
1200
800
400
0
500
375
250
125
0
0
25
50
75
100
TIME AFTER THE INJECTION OF
[1,2-3H] TESTOSTERONE (min)

2. Partial Purification of Nuclear Receptors using Cellulose Phosphate

To determine whether the *in vivo* binding of androgens in nuclei is the same as under *in vitro* conditions, nuclei were examined for peaks of ^{3}H-steroid-protein complexes on cellulose phosphate following the intravenous administration of 150 μCi of [1,2-^{3}H]testosterone to 24 hour castrated rats. After 10, 30 and 60 min the animals were killed and nuclear extracts of prostate were prepared in the usual manner. When these extracts were analyzed on cellulose phosphate columns the results shown in Fig. 5.6 were obtained. Under *in vivo* conditions a single peak of radioactivity was observed and its position in the ionic gradient while differing from the cytosol Peak 2 was identical to the nuclear Peak 2a noted earlier (Fig. 4.9). The ^{3}H-steroid constituents of this peak were dihydrotestosterone and testosterone in the same proportions as shown in Fig. 5.5. Unlike the *in vivo* cytosol binding data, there was a progressive increase in the *size* of this peak at 10, 30 and 60 min as might be expected if the accumulation of androgen labelled protein was dependent upon a rate limiting transfer reaction. No radioactivity corresponding to cellulose phosphate Peak 1 was detected in these experiments.

Fig. 5.6. Demonstration of *in vivo* nuclear receptors on cellulose phosphate. The experimental procedure was identical to that described in the legend to Fig. 4.9. Purified nuclei from 1 g prostate were obtained as described in Chapter II and extracted with Tris-EDTA buffer, pH 7.0 containing 0.6 M NaCl. The extract was applied to a 1 cm x 15 cm column of cellulose phosphate and the receptor complex was eluted with an ionic gradient consisting of Tris-EDTA buffer, pH 7.0 containing 0 to 0.8 M NaCl. Fractions of 4.2 ml each were collected and the radioactivity in each fraction was measured. Radioactivity recovered ●——● 10 min; ○——○ 30 min; ▲——▲ 60 min.


RADIOACTIVITY (cpm/fraction)
500
1000
1500
2000
2500
FRACTION NUMBER
10
20
30
40
50
NaCl CONCENTRATION (m)
0.16
0.32
0.48
0.64
0.80
1
2
2a

3. Chromatography of Nuclear Steroid-Receptors on Sephadex G-200

The *in vivo* labelled nuclear receptors were next examined on Sephadex G-200 columns, 60 and 120 min after the intravenous injection of [1,2-^{3}H]testosterone. The samples were applied to a Sephadex G-200 column and eluted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl. The results shown in Fig. 5.7 reveal that the radioactivity was associated with 2 peaks. The smaller steroid-receptor complex (Stokes' radius ∿ 24 Å) accounted for most of the radioactivity isolated at 60 and 120 min. With increasing time there was a small increase in the amount of label associated with both peaks.

4. The Effects of Freezing on Nuclear Receptors

Because it was not always convenient to analyze *in vivo* labelled nuclear receptors immediately, experiments were performed to determine the effect of freezing on nuclear binding. Castrated rats were injected with 150 μCi of [1,2-^{3}H]testosterone and then after an interval of 60 min were sacrificed. The prostatic nuclei were purified as before, frozen and stored for 24 hours at -10° C. After this period, the nuclei were thawed, extracted and then chromatographed on Sephadex G-200. The results shown in Fig. 5.8 demonstrate that the freezing process drastically alters the character of the nuclear receptor complexes. After freezing, most of the bound radioactivity was eluted in the void volume of the column.

Fig. 5.7. Demonstration of in vivo nuclear receptors on Sephadex G-200. Castrate rats were functionally hepatectomized immediately prior to receiving intravenous injections of 150 Ci of [1,2-^{3}H]-testosterone. Then 60 and 120 min later the rats were sacrificed and nuclei were isolated. Approximately 5 x 10^{7} nuclei from each experiment were extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl and applied to a Sephadex G-200 column (1 cm x 90 cm). Fractions of 1.5 ml were collected after elution of the column with 0.6 M NaCl in buffer. Radioactivity recovered; ●——● , 60 min; ○——○ , 120 min.

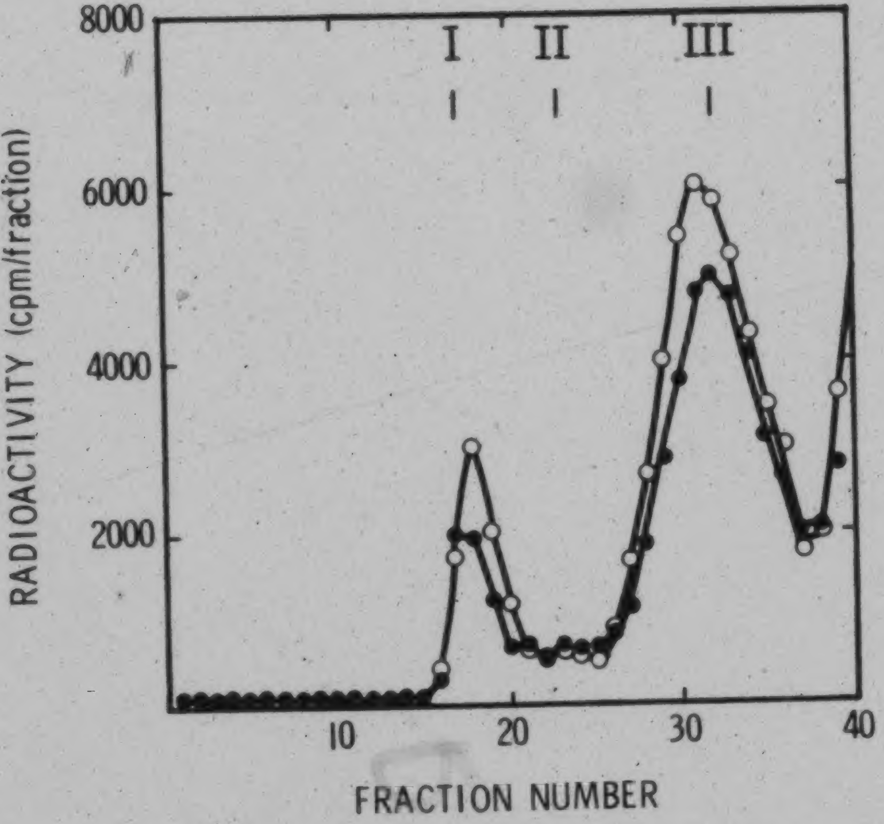
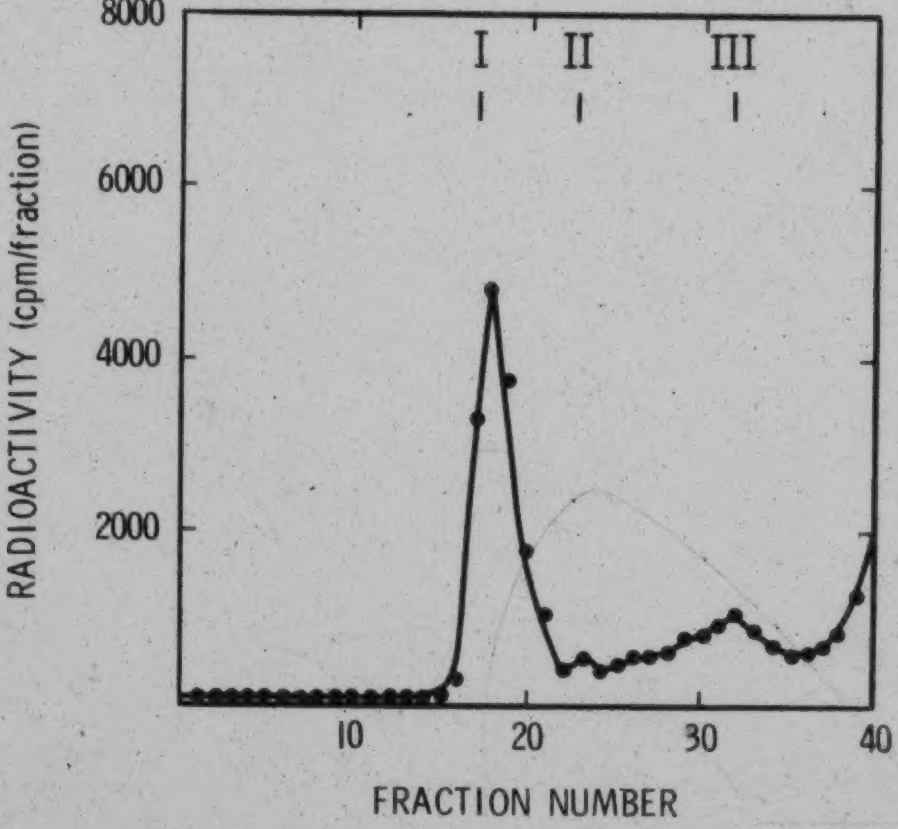

8000
6000
4000
2000
RADIOACTIVITY (cpm/fraction)
I
II
III
10
20
30
40
FRACTION NUMBER

Fig. 5.8. Effect of freezing on intranuclear binding. Approximately 5 x 10^7 nuclei, obtained 60 min after the intravenous injection of [1,2-^{3}H]-testosterone (150 μCi), were frozen for 24 hours. At the end of this period, the nuclei were thawed and extracted with 0.6 M NaCl. The nuclear extract was then applied to a Sephadex G-200 column (1 cm x 90 cm) and eluted with Tris-EDTA buffer, pH 7.0 containing 0.6 M NaCl. Fractions of 1.5 ml were collected and assayed for radioactivity.


8000
6000
4000
2000
RADIOACTIVITY (cpm/fraction)
I
II
III
10
20
30
40
FRACTION NUMBER

Furthermore, the quantity of ^{3}H-steroid associated with this peak increased 2 to 3 fold over the amount seen in this area of the column in control experiments (Fig. 5.7, 60 min). It appears, therefore, that freezing either causes aggregation of receptors or that it enhances the binding of the small intranuclear receptor to chromatin.

D. Discussion

Within 10 min after the injection of [1,2-^{3}H]testosterone into castrated rats the binding of radioactive androgens to cytosol reaches a maximum and then remains nearly constant over a 2 hour period (Fig. 5.1B). The binding is specific in that only dihydrotestosterone and testosterone are bound in spite of the presence of significant quantities of other androgens in the cytoplasm under these conditions (data not shown). On cellulose phosphate the steroid-receptors are recovered in the position of Peak 2 (Fig. 5.2), while on Sephadex G-200 the steroid-receptors are recovered in positions corresponding to Peak I and Peak II (Fig. 5.3). The relative recovery of Peaks I and II is strongly influenced by ionic strength and thus it seems reasonable to believe that both peaks probably represent different forms of the same receptor. A third peak is recovered when cellulose phosphate Peak 2 is incubated in the presence of [1,2-^{3}H]dihydrotestosterone and chromatographed on Sephadex G-200. This peak is shown as Peak III in Fig. 5.4.

The nucleus contains steroid-receptors that chromatograph on cellulose phosphate in a position slightly different from the steroid-receptors of cytosol and accordingly have been defined as Peak 2a receptors to distinguish them from the cellulose phosphate Peak 2 cytosol receptors (Fig. 5.6). On Sephadex G-200 the nuclear receptors are recovered in positions corresponding to Peak I and Peak III (Fig. 5.7) and the relative recovery in Peak I is increased by freezing (Fig. 5.8).

The similar behaviour of Peak 2 and Peak 2a receptors on cellulose phosphate suggests that these molecular entities are closely related. The time dependent increase in radioactivity associated with Peak 2a (Fig. 5.6) indicates that this peak represents the principal intranuclear steroid-receptor. The Sephadex Peak III complex recovered in the nuclei probably corresponds to cellulose phosphate Peak 2a. The fact that the former complex disappears after castration (Fig. 4.10) and is induced by injections of [1,2-^{3}H]testosterone is in keeping with the idea that androgens are transferred from cellulose phosphate Peak 2 of the cytosol to cellulose phosphate Peak 2a (also Sephadex Peak III) of the nucleus. Although the significance of Sephadex Peak I of the nucleus is unclear, several speculative considerations appear worthwhile at this time. Sephadex Peak I may represent an intranuclear site which binds free steroid; equally, it may represent an intranuclear acceptor site that binds steroid-receptor complex; there remains the possibility that it represents aggregates of Sephadex Peak III receptor as suggested by the effects of freezing.

CHAPTER VI

IN VIVO PULSE-CHASE STUDIES

A. Introduction

The studies described in Chapter V indicate that *in vivo* both testosterone and dihydrotestosterone bind to specific cytosol and nuclear receptors. Moreover, it is possible to infer from the results that androgens are transferred from a cellulose phosphate Peak 2 receptor in cytosol to a cellulose phosphate Peak 2a receptor (or Sephadex Peak III receptor) in nuclei. To gain further insight into the process which promotes the incorporation of androgens into nuclei, an *in vivo* pulse-chase procedure was developed. It was expected that this method would provide a means of following the sequential transfer of androgens from the cytoplasmic to the cellular nuclear compartments. If the cytosol receptors migrate from one compartment to the other, one would predict that a chase dose of unlabelled steroid would cause a decrease in the radioactivity associated with previously labelled cytosol receptors; the transfer of a pulse of radioactivity into the nucleus and its subsequent clearance could be followed in a logical sequence. On the other hand, if cytoplasmic binding and incorporation of steroids into nuclei are independent of one another then presumably there would be no correlation between chase-effects on cytosol and nuclei.

Pulse-chase methods were also used to study the action of anti-androgens, such as cyproterone acetate, in displacing natural androgens from steroid-receptors.

B. Materials and Methods

1. Pulse-Chase Procedures

Functionally hepatectomized, castrated rats were injected intravenously with 150 μCi (1 μg) of [1,2-^{3}H]testosterone. Then 10 or 60 min later each animal received an intravenous dose of 250 μg of unlabelled steroid in 250 μℓ of distilled water containing 10% polyoxyethylene sorbitan monopalmitate and 5 to 10% ethanol. Following a second interval of 10 to 120 min the rats were killed and the prostatic tissue was fractionated as described in Chapter II. Binding was determined by gel-filtration on Sephadex G-25 except where stated otherwise.

C. Results

1. Effects of the Size of Pulse on the Incorporation of ^{3}H-Steroids into Prostatic Cytosol and Nuclei

Before pulse-chase studies were conducted it was necessary to evaluate the effectiveness of chase doses of the appropriate steroids. Experiments were performed to measure the concentration of steroid in prostatic cytosol and nuclei after the intravenous injection of [1,2-^{3}H]testosterone, [1,2-^{3}H]dihydrotestosterone and [6,7-^{3}H]estradiol. Both 1 μg (Sp. act. 150 μCi/1 μg) and 250 μg (Sp. act. 150 μCi/250 μg) doses of ^{3}H-steroid were tested and the results are presented in Table 6.1. At 60 min after injection of 1 μg of [1,2-^{3}H]testosterone, 4.01 ± 0.21 pmoles (mean ± S.E.) of ^{3}H-steroid were recovered in the cytosol; after the injection of 1 μg of [6,7-^{3}H]estradiol-17β, 4.24 ± 0.29 pmoles of ^{3}H-steroid were recovered in the same fraction. When radioactive testosterone and estradiol were injected in doses of 250 μg, the amount of ^{3}H-steroid in cytosol increased proportionately to 1250 ± 160 and 1052 ± 68 pmoles respectively. An injection of 250 μg of [1,2-^{3}H]dihydrotestosterone produced a level of 867 ± 41 pmoles which was comparable to the level achieved with 250 μg doses of the other steroids tested. Therefore one might expect that a chase dose of 250 μg of either testosterone or dihydrotestosterone would effect an approximate 250-fold dilution of the metabolites derived from a pulse injection of 1 μg of [1,2-^{3}H]testosterone. Identification of the metabolites of [1,2-^{3}H]-testosterone in the 4 subcellular fractions listed in Table 6.1 revealed that there was no change in the relative amounts of testosterone, dihydrotestosterone and androstanediol after the injection of 1 μg or 250 μg of [1,2-^{3}H]testosterone.

From the data shown in Table 6.1, column 2 it can be seen that the amount of ^{3}H-steroid bound to cytosol protein increases from levels below 1 pmole after the injection of 1 μg of ^{3}H-steroid to levels between 18 and 31 pmoles after the injection of 250 μg of ^{3}H-steroid. While the injection of either [1,2-^{3}H]-testosterone or [1,2-^{3}H]dihydrotestosterone produces similar levels of binding, the injection of [6,7-^{3}H]estradiol supports less binding than the androgens at 1 μg and more binding at 250 μg.

TABLE 6.1

Steroid	Amount Injected (μg)	^{3}H-Steroids Recovered, pmoles/g Wet Wt.			
		Cytosol	Cytosol Receptor	Nucleus	Nuclear Receptor
Testosterone	1 μg	4.01 ± 0.21	0.70 ± 0.08	9.20 ± 1.14	4.05 ± 0.30
Testosterone	250 μg	1250 ± 160	19.00 ± 2.00	18.10 ± 3.10	6.02 ± 0.20
Dihydrotestosterone	1 μg	-	-	-	-
Dihydrotestosterone	250 μg	867 ± 41	18.40 ± 1.00	11.83 ± 0.31	4.74 ± 0.51
Estradiol-17β	1 μg	4.24 ± 0.29	0.18 ± 0.02	0.22 ± 0.03	0.06 ± 0.01
Estradiol-17β	250 μg	1052 ± 68	30.90 ± 5.80	10.00 ± 3.01	1.65 ± 0.32

Effect of Size of Pulse on the Incorporation of ^{3}H-Steroids into Prostatic Cytosol and Nuclei - Groups of 3 to 5 rats castrated 24 hours previously were injected intravenously with either 1 μg (150 μCi/μg) or 250 μg (150 μCi/250 μg) of [1,2-^{3}H]testosterone, [1,2-^{3}H]-dihydrotestosterone and [6,7-^{3}H]estradiol. 60 min later the animals were killed and the appropriate cytosol and nuclear fractions were prepared as described in Chapter II and assayed for radioactivity. The results are presented as the mean ± S.E. for each set of at least 3 replicate experiments.

The effect of dose on the uptake and binding of steroids by nuclei is shown in Table 6.1, columns 3 and 4. Levels of 9.20 ± 1.14 pmoles and 4.05 ± 0.30 pmoles were established for incorporation and binding respectively by the injection of 1 μg of [1,2-^{3}H]testosterone. These levels increased slightly to 18.10 ± 3.10 pmoles and 6.02 ± 0.20 pmoles after the injection of 250 μg of [1,2-^{3}H]testosterone. However, the approximate doubling of the values represents a diminutive response in comparison to the 300-fold increase in the concentration of cytoplasmic steroids. Undoubtedly the results reflect the considerable difference in the permeability of plasma and nuclear membranes. Whereas there is little or no restriction to the diffusion of androgens across the plasma membrane, the transfer of these compounds across the nuclear membrane is decidedly limited.

Doses consisting of 250 μg of [1,2-^{3}H]dihydrotestosterone or of [6,7-^{3}H]estradiol produced levels of ^{3}H-steroid in nuclei of 11.83 ± 0.31 pmoles and 10.00 ± 3.01 pmoles respectively (Table 6.1, column 3), slightly lower than the levels produced by [1,2-^{3}H]-testosterone. It is clear therefore that estradiol is incorporated by nuclei as efficiently as dihydrotestosterone at the higher dose. The amount of binding after the injection of 250 μg of [1,2-^{3}H]dihydrotestosterone at 4.74 ± 0.51 pmoles was approximately the same as that achieved with an injection of [1,2-^{3}H]testosterone but about 1/2 to 1/3 of that amount of binding was produced by the injection of 250 μg of [6,7-^{3}H]estradiol.

2. Effect of Chasing on the Incorporation of ^{3}H-Androgens into Cytosol

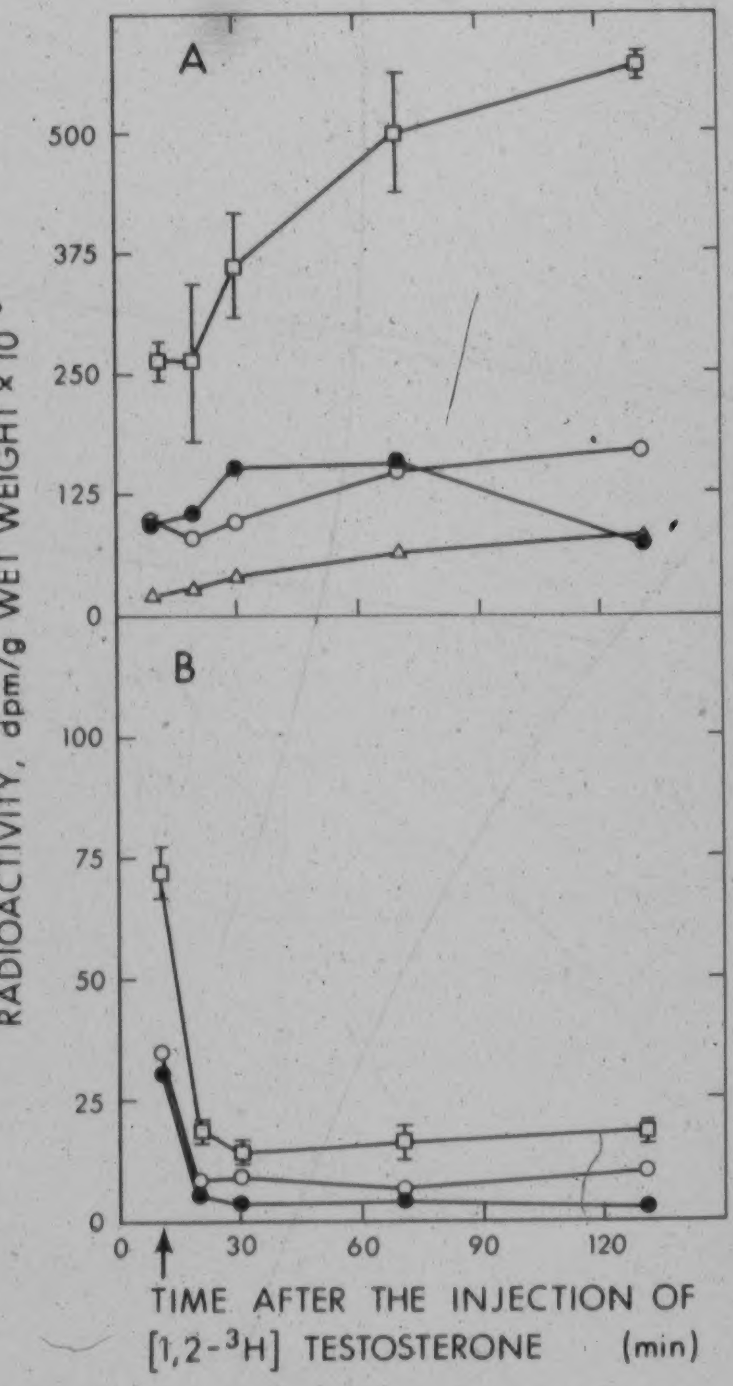
Experiments were next performed to investigate the turnover of ^{3}H-steroids in cytoplasm and nuclei. Rats castrated 24 hours previously were injected with 150 μCi (1 μg) of [1,2-^{3}H]testosterone. This injection was followed 10 min later by a second dose or 250 μg of unlabelled testosterone. At intervals of 10, 20, 60 and 120 min after the second dose, the rats were killed and the prostatic tissue was fractionated as before.

The effect of the chase procedure on the cytosol fraction is shown in Fig. 6.1A (upper curve). The uptake of radioactivity into cytosol was not unlike that observed in pulse experiments (Fig. 5.1A), and no striking departures in the relative amounts of the three principal metabolites were evident.

As shown in Fig. 6.1B the ^{3}H-androgens bound to cytosol protein display a greater sensitivity to a chase dose of testosterone than does the complete cytosol fraction. Within 10 min following the administration of unlabelled testosterone an amount of radioactive steroid equivalent to 55×10^{3} dpm is lost from the ^{3}H-steroid-receptor fraction.

When cytosol extracts from pulse-chase experiments were examined on cellulose phosphate columns the amount of radioisotope recovered in association with proteins was extremely small and was confined entirely to the Peak 2 area. In qualitative respects, therefore, the cellulose phosphate chromatograms were similar to those

Fig. 6.1. Effect of chasing on the incorporation of ^{3}H-androgens into cytosol. Rats castrated 24 hours previously were functionally hepatectomized and injected with 150 µCi (1 µg) of [1,2-^{3}H]testosterone. This injection was followed 10 min later by a second injection of 250 µg of unlabelled testosterone as marked by the arrow on the abscissa. At intervals of 10, 20, 60 and 120 min after the second dose, the rats were killed and the prostatic tissue was fractionated as described in Chapter II. Cytosol and cytosol receptor fractions were assayed for radioactivity and metabolites were identified. Panel A, radioactivity and metabolites recovered in cytosol; panel B, in cytosol receptor. Total radioactivity (mean ± S.E.), □——□ ; dihydrotestosterone ○——○ ; testosterone ●——● ; androstanediol △——△ .


A
B
RADIOACTIVITY, dpm/g WET WEIGHT × 10⁻³
500
375
250
125
0
100
75
50
25
0
30
60
90
120
TIME AFTER THE INJECTION OF [1,2-³H] TESTOSTERONE (min)

obtained in pulse experiments (Fig. 5.2). Examination of cytosol receptors on Sephadex G-200 columns, however, revealed that chasing not only reduces the quantity of bound radioactivity but also alters the distribution of ^{3}H-steroids (Fig. 6.2). In contrast to the results obtained in pulse experiments (Fig. 5.3), in which no binding was detected in Sephadex Peak III, trace amounts of this type of receptor were labelled in pulse-chase experiments. The results shown in Fig. 6.2 thus imply that Sephadex Peak II (or Peak I) receptor gives rise to Sephadex Peak III receptor *in vivo*.

3. Effects of Chasing on the Incorporation of ^{3}H-Androgens into Nuclei

The effect of a chase injection of unlabelled testosterone on the incorporation of ^{3}H-androgens into nuclei was studied next and the results are plotted in Fig. 6.3A. Except for the small initial increase from 195 x 10^3 dpm at 10 min to 245 x 10^3 dpm at 30 min, the uptake of radioactivity was rapidly and completely inhibited by the administration of chase (compare to Fig. 5.5A). Moreover, the constancy of the amount of radioactive label retained by nuclei between 30 min and 130 min indicates that there was no flux of pulse-derived ^{3}H-androgens into or out of the nucleus. The reciprocal change in the amount of testosterone and dihydrotestosterone and the slight accumulation of dihydrotestosterone with increasing time are presumably manifestations of the intranuclear metabolism of testosterone (Fig. 6.3A, lower curves).

Fig. 6.2 Chromatography of cytosol steroid-receptors on Sephadex G-200 after pulse-chase sequence. Rats castrated 24 hours previously were functionally hepatectomized and then injected with 150 μCi (1 μg) [1,2-^{3}H]testosterone. After 10 min the animals received an intravenous injection of unlabelled testosterone (250 μg), and were sacrificed after a further 20 min period. Cytosol, isolated from 1 g of prostate, was precipitated with ammonium sulphate (80% saturation) and then applied to a Sephadex G-200 column (1 cm x 90 cm). Fractions of 1.5 ml were eluted from the column with Tris-EDTA buffer, pH 7.0, and the radioactivity in each was measured.

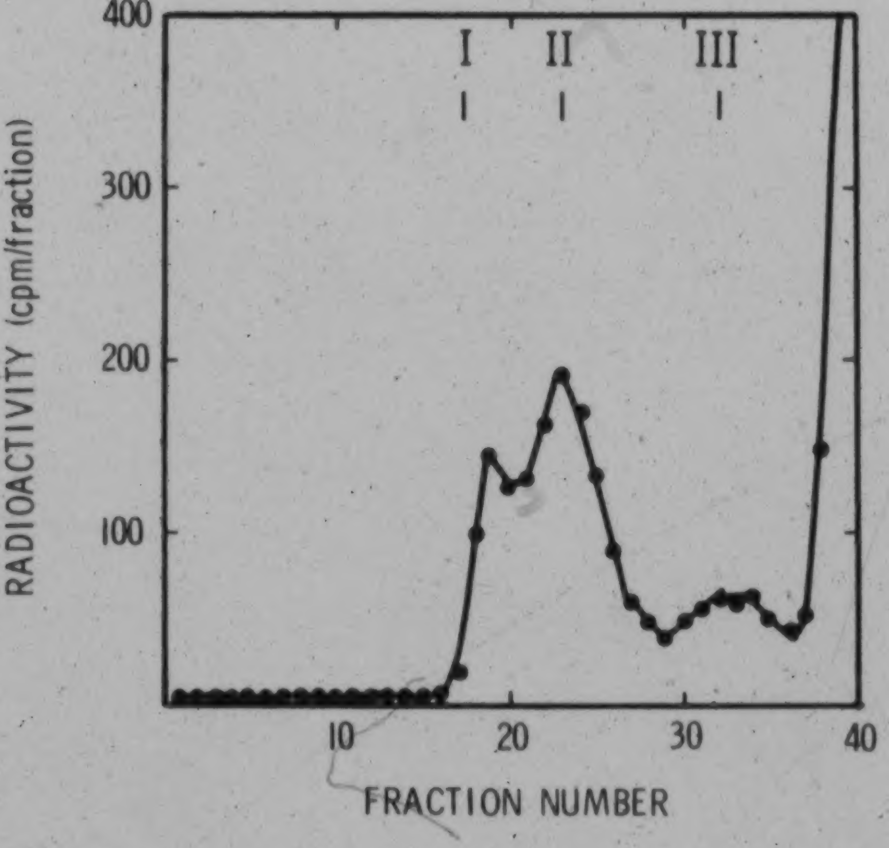
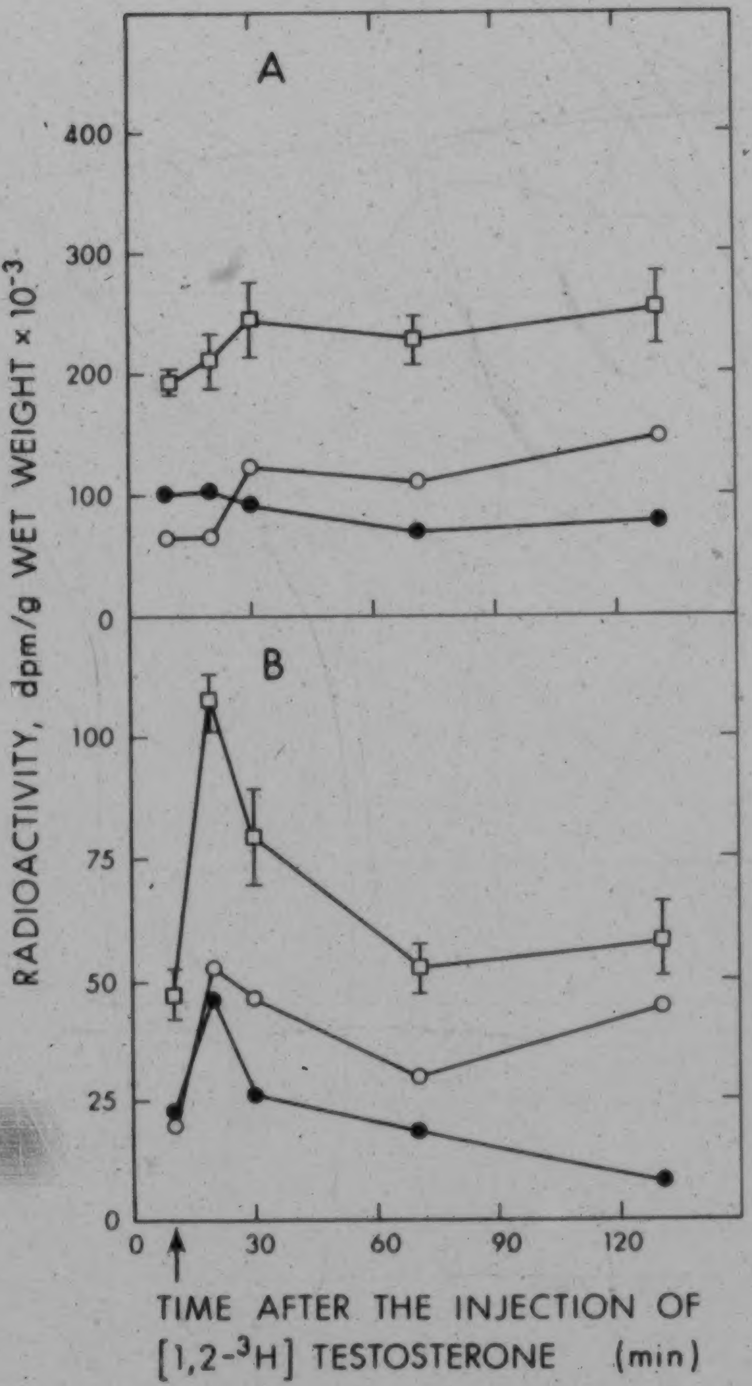

400
300
200
100
10
20
30
40
I
II
III
RADIOACTIVITY (cpm/fraction)
FRACTION NUMBER

Fig. 6.3. Effect of chasing on the incorporation of ^{3}H-androgens into nuclei. Rats were treated as described in the legend to Fig. 6.2. Purified nuclei were extracted and analysed as described in Chapter II. Fractions containing nuclei and nuclear receptor were assayed for radioactivity and metabolites were identified. Panel A, radioactivity and metabolites recovered in intact nuclei; panel B, in nuclear receptor. Total radioactivity (mean ± S.E.) □———□ ; dihydrotestosterone, ○———○ ; testosterone ●———● .


A
B
RADIOACTIVITY, dpm/g WET WEIGHT × 10⁻³
400
300
200
100
0
100
75
50
25
0
0
30
60
90
120
TIME AFTER THE INJECTION OF
[1,2-³H] TESTOSTERONE (min)

The outcome of a chase injection of unlabelled testosterone on nuclear ^{3}H-steroid-receptor was examined and a much different result was obtained as shown in Fig. 6.3B (upper curve). During the 10 min immediately following the administration of chase, the level of ^{3}H-steroid in the receptor fraction increased from 48 x 10^3 dpm to 108 x 10^3 dpm representing a net accumulation of 60 x 10^3 dpm. Immediately following the peak at 20 min the level of ^{3}H-steroid in the receptor fraction declined rapidly to 53 x 10^3 dpm at 70 min and thereafter did not change significantly. The decline in binding of ^{3}H-androgens appears to result from the displacement of both testosterone and dihydrotestosterone as can be seen from the lower curves of Fig. 6.3B. Beyond the 70 min interval, however, more testosterone was displaced than dihydrotestosterone and the relative amount of the latter in the receptor fraction actually increased. The striking similarity in the amount of ^{3}H-androgens lost from cytosol ^{3}H-steroid-receptor complex (55 x 10^3 dpm) and simultaneously gained by the nuclear ^{3}H-steroid-receptor complex (60 x 10^3 dpm) and by isolated nuclei (50 x 10^3 dpm) constitutes strong albeit indirect evidence that ^{3}H-steroid has been transferred from one receptor to the other and that the incorporation of androgenic compounds by nuclei is wholly dependent on such a transfer reaction.

With reference to the data in Table 6.1, columns 3 and 4 (injection of 250 μg of testosterone) it is possible to estimate the dilution of specific radioactivity of the pulse-derived ^{3}H-steroid caused by the chasing procedure. For example, 1 hr after the chase injection of testosterone the total amount of steroid in nuclei is 18.10 pmoles while the amount of radioactive steroid in nuclei is only 2.25 pmoles. Similarly the amount of steroid in the receptor fraction is 6.02 pmoles but only 0.53 pmoles is present as radioactive material. From these values it is clear that the chasing procedure reduces the specific radioactivity of the pulse derived ^{3}H-steroid 8 to 11 fold in isolated nuclei and in the steroid-receptor complex. Since there appears to be a restriction on the amount of steroid taken up by nuclei, this dilution of specific radioactivity probably represents the maximum that can be achieved under the experimental conditions described. The absence of further dilution of specific radioactivity may explain why the amount of ^{3}H-steroid in the receptor fraction at 70 and 130 min is the same (Fig. 6.3B).

Nuclear extracts obtained after a 20 min chase interval were chromatographed on cellulose phosphate. The results of this analysis are shown in Fig. 6.4. After a 10 min pulse with [1,2-^{3}H]-testosterone (1 μg) and a 20 min chase with unlabelled testosterone (250 μg), most of the radioactivity was recovered in Peak 2a and a small amount in Peak 1. Thus after a short chase interval most of the binding was to the carrier form of receptor.

To investigate this phenomenon further, nuclear extracts isolated after the pulse-chase sequence were chromatographed on Sephadex G-200. Fig. 6.5 shows the radioactive profile observed when nuclear extracts obtained after a 60 min chase were examined in this fashion. More ^{3}H-steroid is recovered in Peak I (void volume) of the

Fig. 6.4. Chromatography of nuclear receptors on cellulose phosphate after pulse-chase sequence. Rats, castrated 24 hours previously, were functionally hepatectomized, and injected with 150 μCi (1 μg) of [1,2-^{3}H]testosterone. After 10 min the animals received an intravenous injection of unlabelled testosterone (250 μg). After a chase interval of 20 min the rats were killed and the prostatic nuclei were isolated. Approximately 5 x 10^7 nuclei were extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl, desalted, and applied to cellulose phosphate columns (1 cm x 15 cm). The elution of proteins was performed in the same manner as in pulse experiments with an ionic gradient consisting of Tris-EDTA buffer, pH 7.0, containing 0 to 0.8 M NaCl. Fractions of 4.2 ml were collected and the radioactivity in each was measured.

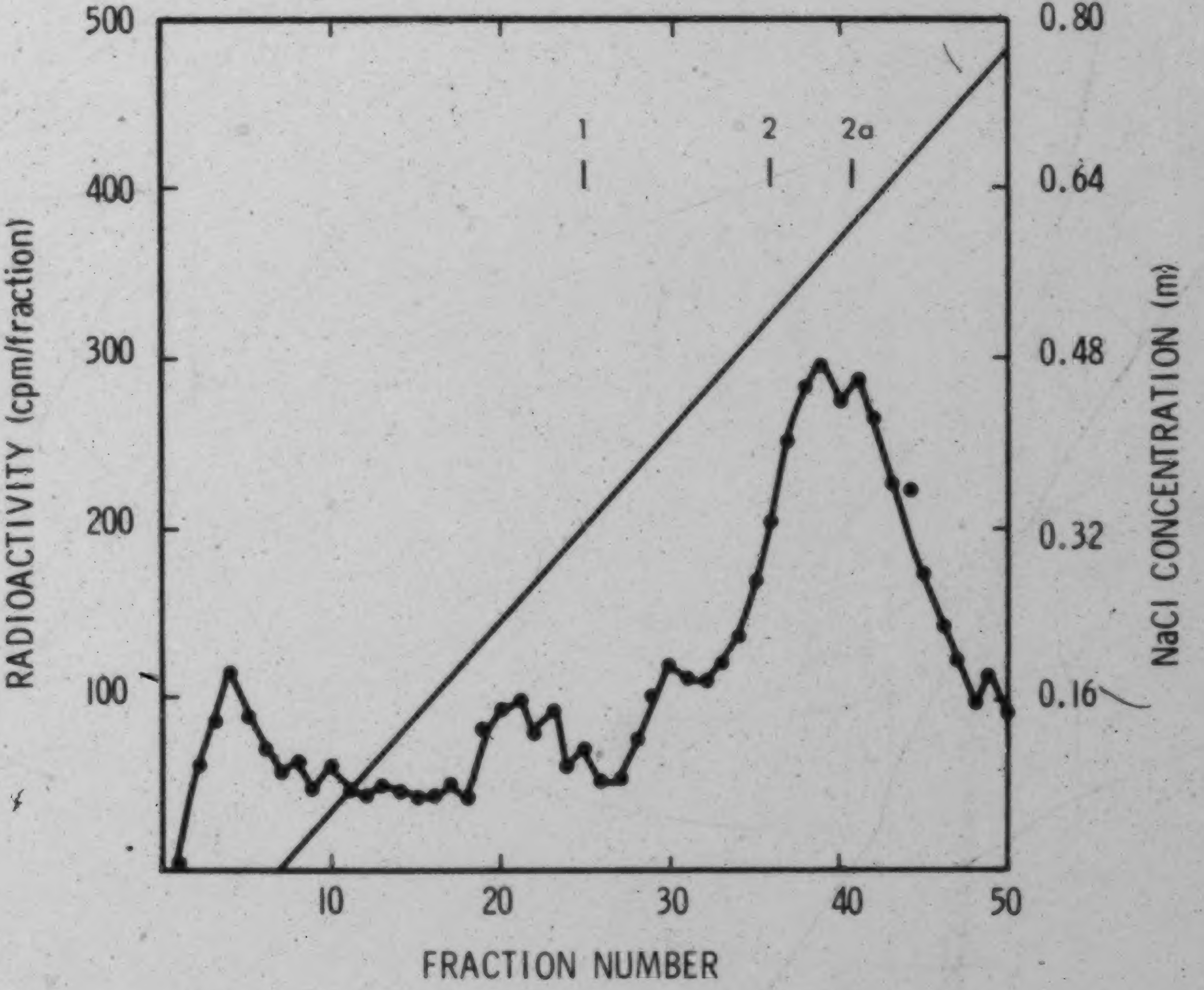
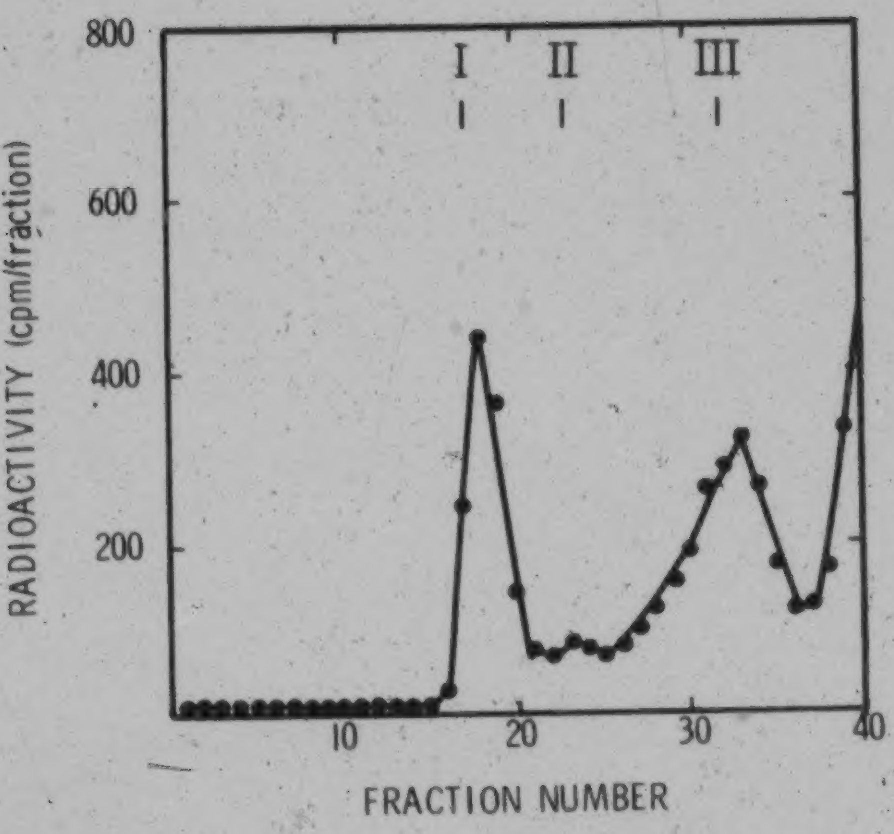

RADIOACTIVITY (cpm/fraction)
500
400
300
200
100
NaCl CONCENTRATION (m)
0.80
0.64
0.48
0.32
0.16
1
2
2a
10
20
30
40
50
FRACTION NUMBER

Fig. 6.5. Chromatography of nuclear receptors on Sephadex G-200 after pulse-chase sequence. Castrate rats were functionally hepatectomized and injected with 150 μCi of [1,2-^{3}H]testosterone (1 μg); 10 min later each was injected with unlabelled testosterone (250 μg). After a further 60 min the rats were killed and the prostatic nuclei isolated. Approximately 5 x 10^{7} nuclei were extracted with Tris-EDTA buffer, pH 7.0, containing 0.6 M NaCl, and applied to a Sepahdex G-200 column (1 cm x 90 cm). Fractions of 1.5 ml were eluted with the above NaCl solution and the radioactivity in each was determined.


800
600
400
200
RADIOACTIVITY (cpm/fraction)
I
II
III
10
20
30
40
FRACTION NUMBER

column than in Peak III. When these data are compared to those obtained in pulse experiments (Fig. 5.7) it is apparent that under chase conditions the relative amounts of radioactivity in the two peaks shifts in favour of the larger molecular species.

4. Effects of Extending the Pulse Interval

In previous pulse-chase experiments, a pulse interval of 10 min proved sufficient to label nuclei and nuclear binding protein and under the conditions of the experiments no release of ^{3}H-steroid from the nucleus was detected (see Fig. 6.3A). Further experiments were carried out in order to determine whether discharge could be demonstrated by allowing more ^{3}H-steroid-receptor to accumulate in nuclei before the administration of chase. The effect of extending the pulse interval to 60 min is shown in Table 6.2. From the data listed in column 1 it is evident that incorporation of ^{3}H-steroid into nuclei after 60 min of chase (1023 x 10^3 dpm) was slightly above the value attained after 60 min of pulse (920 x 10^3 dpm) although the results were not statistically different; the mean recoveries of ^{3}H-steroid in the receptor fraction at corresponding times also did not differ. Qualitatively these results are similar to those reported in Fig. 6.3A, and again the attempt to demonstrate discharge of ^{3}H-steroids from the nucleus was not successful.

Examination of the metabolites recovered in the various fractions of prostate (Table 6.2, columns 2 and 3) indicate that following chase the amount of testosterone in nuclei and the nuclear receptor fraction was substantially below the 60 min and 120 min

TABLE 6.2

Fraction	Experiment		Radioactivity recovered in sample, dpm/g x 10^{-3}		
	Pulse (min)	Chase (min)	Total	Testosterone	Dihydrotestosterone
Nucleus[1]	60		920 ± 114	429 ± 53	399 ± 50
	120		1512 ± 142	493 ± 48	850 ± 80
	60	60	1023 ± 62	180 ± 15	822 ± 51
Nuclear Receptor[1]	60		405 ± 30	126 ± 23	252 ± 26
	120		474 ± 47	142 ± 47	293 ± 30
	60	60	399 ± 74	54 ± 10	337 ± 63

Effect of Extending the Pulse Interval to 60 Min - Groups of 3 to 5 rats castrated 24 hours previously were injected intravenously with 1 μg (150 μCi) of [1,2-^{3}H]testosterone. Prostatic nuclei from control groups were assayed 60 min and 120 min after the injection of pulse. Remaining rats were injected with 250 μg of unlabelled testosterone 60 min after the injection of pulse and the prostatic nuclei were assayed 120 min after pulse. Nuclei were isolated as described in Chapter II and binding was measured by gel-filtration on Sephadex G-25. Steroids in each fraction were identified by thin-layer chromatography. The results are expressed as the mean ± S.E. of at least 3 replicate experiments.

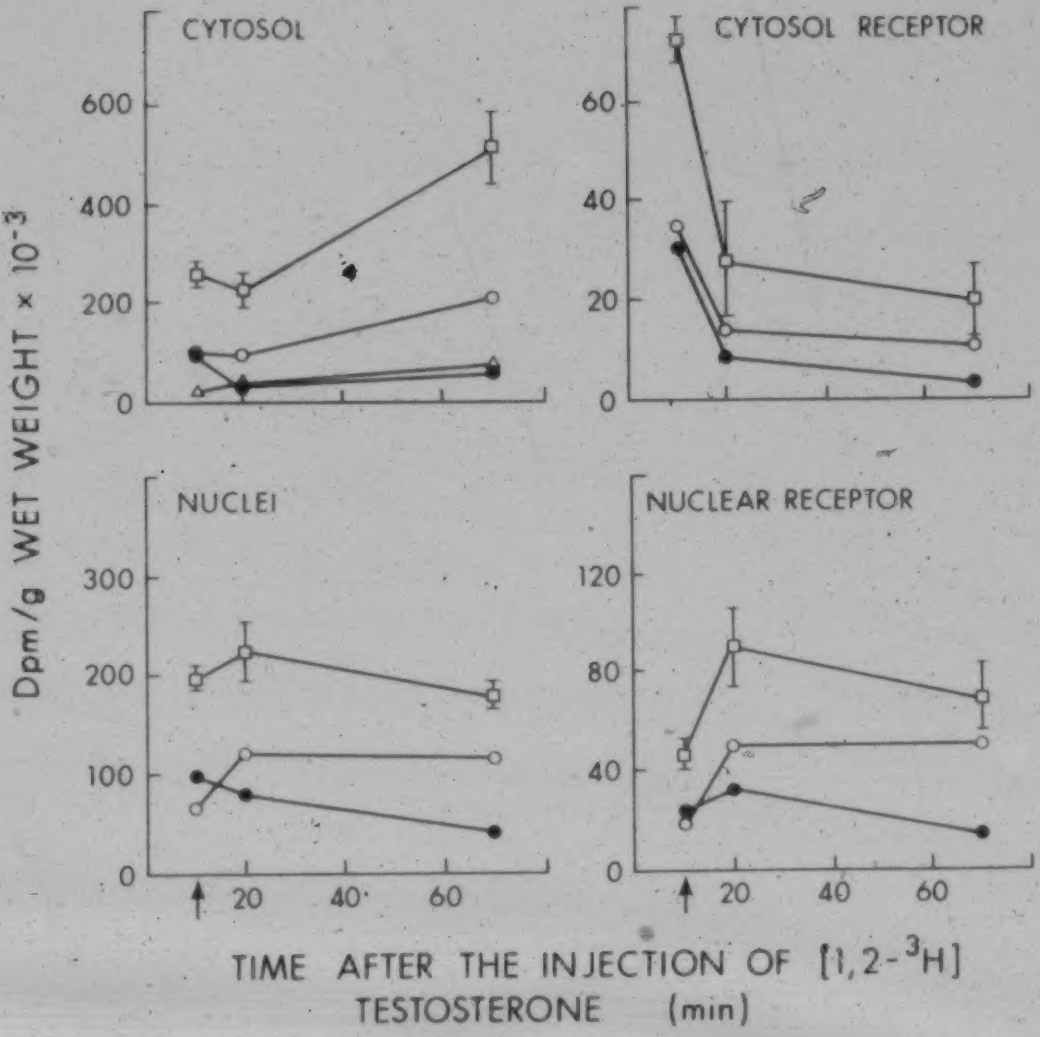
[1] The values for the 60 and 120 min pulse were obtained from Fig. 5.5.

control levels. As there was no demonstrable loss of ^{3}H-steroid from the nucleus during the experiment, it appears reasonable to believe that testosterone was metabolized to dihydrotestosterone accounting for the increase in the ratio of dihydrotestosterone: testosterone from 1:1 after a pulse interval of 60 min to more than 4:1 after the pulse-chase sequence. An alternative but less likely explanation is that the increased dihydrotestosterone associated with the nuclear fractions results from the selective uptake of this steroid accompanied by the coincident loss of testosterone from the nucleus.

5. Effect of Chasing with Dihydrotestosterone on the Incorporation of ^{3}H-Androgens into Cytosol and Nuclei

Since dihydrotestosterone is a highly potent androgen in rat prostate and since it is also the predominant metabolite in prostate, experiments were conducted to determine whether or not dihydrotestosterone is a more effective chase material than testosterone. Rats castrated 24 hours previously were injected with 150 μCi (1 μg) of [1,2-^{3}H]testosterone and 10 min later were injected with 250 μg of unlabelled dihydrotestosterone. After further intervals of 10 and 60 min the rats were killed and appropriate cytosol and nuclear fractions were assayed in the usual manner. Fig. 6.6 shows the results of these experiments. An immediate but transient arrest of ^{3}H-steroid uptake occurred in cytosol and the level of radioactivity at 70 min was almost identical to

Fig. 6.6. Effect of chasing with dihydrotestosterone. Rats were treated as described in the legend to Fig. 6.3, except that the chase injection was dihydrotestosterone instead of testosterone. Cytosol and nuclear fractions were assayed for radioactivity and metabolites were identified by thin-layer chromatography. Total radioactivity, (mean ± S.E.), □——□ ; dihydrotestosterone, ○——○ ; testosterone ●——● ; androstanediol △——△ .


CYTOSOL
CYTOSOL RECEPTOR
NUCLEI
NUCLEAR RECEPTOR
Dpm/g WET WEIGHT × 10-3
600
400
200
0
60
40
20
0
300
200
100
0
120
80
40
0
20
40
60
20
40
60
TIME AFTER THE INJECTION OF [1,2-3H] TESTOSTERONE (min)

the level reached when testosterone was used as chase (Fig. 6.1). At this time the amounts of testosterone and dihydrotestosterone in cytosol were also virtually the same as the amounts obtained in the experiment with testosterone. Dihydrotestosterone caused an initial sharp reduction in the amount of ^{3}H-steroid associated with cytosol receptor and as expected there was a reciprocal increase in the amount of ^{3}H-steroid in the nuclear receptor fraction. Although the 10, 20, and 70 min levels of ^{3}H-steroid in nuclei did not differ significantly, the mean level increased slightly during the 10 min period immediately following the injection of chase. Dihydrotestosterone was less effective than testosterone in displacing ^{3}H-steroid from the nuclear receptor between 20 min and 70 min. On the other hand, the tendency towards disappearance of testosterone from nuclei and from nuclear receptor was comparable to the trend observed in Fig. 6.4, and in Table 6.2 with testosterone. In the present case, however, the significance of this observation is less certain since the small decline in the mean level of nuclear radioactivity over the duration of the experiment is consistent with the possibility that testosterone is selectively released from the nucleus following a chase injection of dihydrotestosterone.

6. Effects of Estradiol, Cyproterone Acetate and Epitestosterone on the Intracellular Transport of Androgens

It has been suggested that estradiol may antagonize the action of androgens at the cellular level by inhibiting the conversion of testosterone to dihydrotestosterone (Shimazaki *et al.*, 1965; Farnsworth, 1969; Groom et al., 1971; Leav et al., 1971), or by preventing the association of androgens to cytosol receptors (Fang et al., 1969). Cyproterone acetate is another agent which induces an involutional effect on the growth and function of male accessory reproductive tissues (Neumann and Von Berswordt-Walbrabe, 1966) and is thought to cause this effect through competitive inhibition of the intracellular binding of androgens (Stern and Eisenfeld, 1969; Belham and Neal, 1971; Mangan and Mainwaring, 1972). Epitestosterone is a relatively inactive androgen but has been reported to inhibit the metabolism of testosterone in vitro (Frederiksen and Wilson, 1971). Each of these compounds was tested for capacity to interfere with the transfer of steroids from cytosol receptor to nuclear receptor. The results of experiments in which the pulse injection of 150 μCi (1 μg) of [1,2-^{3}H]testosterone was succeeded 10 min later by a chase injection of 250 μg of test compound are shown in Table 6.3. Values obtained 60 min after the administration of chase were compared with values expected in the absence of chase as obtained from Fig. 5.1 and Fig. 5.5.

The mean levels of ^{3}H-androgens in cytosol after chase injections of estradiol, epitestosterone, and cyproterone acetate were identical and did not differ significantly from the mean level after the chase injection of testosterone. However, the amount of ^{3}H-steroid in the cytosol receptor fraction was reduced by 54%, 39%, and 54% relative to control following chase injections of estradiol,

TABLE 6.3

Chase Injection	Radioactivity Recovered							
	Cytosol		Cytosol Receptor		Nucleus		Nuclear Receptor	
	Dpm/g x 10^3	% Change	Dpm/g x 10^3	% Change	Dpm/g x 10^{-3}	% Change	Dpm/g x 10^{-3}	% Change
Testosterone[1]	504 ± 64	+ 22	16 ± 4	- 78	227 ± 20	- 87	53 ± 4	- 87
17β-Estradiol	445 ± 62	+ 7	32 ± 7	- 54	620 ± 46	- 41	264 ± 27	- 36
Epitestosterone	444 ± 38	+ 7	43 ± 6	- 39	509 ± 17	- 51	232 ± 25	- 46
Cyproterone-Acetate	440 ± 32	+ 6	32 ± 3	- 54	606 ± 28	- 42	199 ± 10	- 52

Pulse-Chase Experiments with Cyproterone Acetate and Epitesterone - Groups of 3 to 5 rats castrated 24 hours previously were injected intravenously with 1 μg (150 μCi) of [1,2-^{3}H]testosterone. Ten min later the animals received a second intravenous injection containing 250 μg of unlabelled testosterone, estradiol, epitestosterone or cyproterone acetate. Sixty min after the chase injection, the rats were killed and fractions of prostatic cytosol and nuclei were isolated. Binding was measured by gel-filtration. The results are expressed as the mean ± S.E. of at least 3 replicate experiments and as the percentage change from the control values established in pulse experiments described elsewhere in this report.

[1] The values for the experiments with testosterone were obtained from Fig. 6.1 and 6.3.

epitestosterone, and cyproterone acetate respectively; none of these compounds was as effective as testosterone which produced a reduction of 78%. From the remaining data in Table 6.3 it is evident that the reduction of ^{3}H-steroid in nuclei and in the nuclear receptor fraction almost parallels the reduction of ^{3}H-steroid in the cytosol receptor fraction and again it is clear that the most profound changes are induced by testosterone. A final point that merits emphasis is that the reduction of ^{3}H-androgens in nuclear receptor caused by estradiol is equivalent to 1.49 ± 0.10 pmoles which is virtually the same quantity of estradiol recovered in this fraction following the pulse administration of 250 µg of [6,7-^{3}H]estradiol (Table 6.1). This result would be predicted if estrogens and androgens were bound to the same nuclear receptor.

The metabolism of [1,2-^{3}H]testosterone was examined in this series of experiments as well and no significant change was observed in the recovery of radioactive testosterone, dihydrotestosterone, and androstanediol after the chase injection of any of the test compounds listed in Table 6.3. Consequently the antagonistic action of estradiol and cyproterone acetate at the cellular level is probably not expressed through any alterations in the activity of enzymes involved in androgen metabolism. Rather, it is more likely that such action is explained on the basis of inhibition of the binding of testosterone and dihydrotestosterone to cytosol and nuclear receptors. The behaviour of epitestosterone is compatible with that of an androgen antagonist insofar as it also inhibits both the binding of testosterone and dihydrotestosterone to cytosol receptor and the transport of these steroids into the nucleus.

7. The Metabolism of [6,7-^{3}H]Estradiol in Rat Prostate

The metabolites of [6,7-^{3}H]estradiol appearing in prostatic cytosol and cytosol receptor 60 min after the injection of 1 μg and 250 μg of steroid were identified in order to assess the possibility that the inhibitory action of estradiol is promoted by another estrogenic compound. As shown in Table 6.4 more estrone than estradiol was recovered in the cytosol and cytosol receptor fractions. A small amount of estriol was present as was a significant amount of unidentified material described as "other". Preliminary analyses of ^{3}H-steroid recovered in nuclei suggest that only estradiol is present in the nuclear fraction. On the basis of available information no judgement can be reached as to the contribution of estrone to the anti-androgenic effect of estradiol; in this respect, however, it is clear that the potential action of estrone should not be overlooked.

D. Discussion

In these experiments the transport of androgens in rat prostate was studied *in vivo* with the use of a pulse-chase method. Fractions of cytosol and nuclei were labelled by a pulse injection of 150 μCi (1 μg) of [1,2-^{3}H]testosterone and this operation was followed by a chase injection of 250 μg of unlabelled testosterone.

TABLE 6.4

Fraction	Estradiol Injected (µg)	Estrogens Recovered in Neutral Steroid Fraction (% of Total Radioactivity)			
		Estradiol	Estrone	Estriol	Other
Cytosol	1	14.7	58.7	3.9	22.7
	250	34.5	39.0	1.5	25.0
Cytosol Receptor	1	26.3	37.8	2.3	33.6
	250	20.4	41.0	1.2	37.4

The Metabolism of $[6,7-^3H]$Estradiol in Rat Prostate - Groups of 3 to 5 rats castrated 24 hours previously were injected intravenously with 1 µg (150 µCi) or 250 µCi (150 µCi) of $[6,7-^3H]$estradiol. 60 min later the rats were killed and fractions of cytosol and nuclei were isolated. These were extracted with chloroform-methanol (2:1, v/v) and the steroid composition was determined by thin-layer chromatography. The results are expressed as the percentage of the total radioactivity in the neutral steroid fraction recovered in each steroid listed. Duplicate, and in some cases triplicate, experiments were performed and the average value was calculated.

In most experiments the first and second injections were separated by a 10 min interval. It was calculated that this experimental design yielded a significant reduction in specific radioactivity of pulse derived ^{3}H-steroid, namely an approximate 250-fold dilution in the cytosol and an 8 to 11-fold dilution in the nucleus 60 min after the administration of chase. Since there was no change in the relative amounts of testosterone, dihydrotestosterone and androstanediol after the injection of 1 μg or 250 μg of [1,2-^{3}H]testosterone it was reasonably certain that the degree of dilution of intracellular androgens derived from testosterone was similar.

A large difference between the permeability of the plasma and nuclear membranes was demonstrated by two findings. First, the amount of steroid incorporated by cytosol was proportional to the dose of steroid injected whereas no direct relationship was found between dose and uptake of steroids by nuclei (Table 6.1). Secondly, the apparent dilution of specific radioactivity of pulse derived ^{3}H-steroid in nuclei was greatly exceeded by the dilution in cytosol (Fig. 6.1 and 6.3). It seems certain, therefore, that the passage of steroids through the plasma membrane is explained in terms of a passive diffusion mechanism as has been assumed in the past (Giorgi et al., 1971; Munck, 1971). The passage of steroids through the nuclear membrane, however, is identifiable as an active transport mechanism on the basis of both the relative saturability of the system (Table 6.1) and the accumulation of steroid in nuclei against a chemical gradient. The latter result can be deduced from examination of the data presented in Figs. 5.1 and 5.5 of Chapter V which clearly showed that at 30, 60 and 120 min after the injection of 150 μCi (1 μg) of [1,2-^{3}H]testosterone the amount of ^{3}H-androgen in nuclei steadily increased over the amount in the cytoplasm. This evidence along with the inability to demonstrate counter-transport (Fig. 6.3 and Table 6.2) rules out the possibility that steroids are transferred into the nucleus by facilitated diffusion. Two additional findings are in keeping with active transport, namely that testosterone and dihydrotestosterone are transferred quantitatively from a cytosol receptor to a nuclear receptor of slightly different structure (compare Peak 2 and Peak 2a; also Fang _et al_., 1969) and that these steroids are released from the steroid-receptor complex after penetrating the nucleus (Fig. 6.3B). In effect it would seem that the steroid-receptor complex represents a true carrier-substrate complex formed on one side of the membrane and modified on the other in such a way that the carrier has lowered affinity for its substrate (Stein, 1967; Kaback, 1972). It is also tempting to suggest that the apparent trapping or retention of steroids by the nucleus results from the conversion of steroid from a bound, transportable form to a free, non-transportable form. No judgement can be reached, however, as to whether steroids exist in a free form as such or whether they are bound to low-affinity intranuclear receptors. Neither is there sufficient data to assess the possibility that the carrier returns to the cytoplasm to initiate another transport cycle as has been proposed for the transport of glucocorticoids (Munck _et al_., 1972; Ishii _et al_., 1972).

The dilution of the specific radioactivity of intranuclear ^{3}H-steroid in pulse-chase experiments results in the partial disappearance of ^{3}H-steroid from the steroid-receptor complex (Fig. 6.3B). This effect is probably related to the intranuclear mixing of labelled and unlabelled steroid and provides indirect proof of exchange of steroids between a bound and free compartment. Further suggestive evidence of a free compartment is obtained from the results of experiments on the incorporation of ^{3}H-androgens by nuclei after the pulse injection of [1,2-^{3}H]testosterone shown in Chapter V, Fig. 5.5. From a comparison of Fig. 5.5A and Fig. 5.5B it is readily apparent that the fraction of intranulcear steroid recovered in the nuclear receptor fraction is close to 30% (mean of 25%, 27%, 43%, and 32% is equal to 32%) at each time point indicated. Since this fraction never exceeds 43% of the total steroid in the nucleus the question arises as to whether the unbound steroid originates anomalously from the breakdown of steroid-protein complex during gel-filtration or whether indeed it represents a physiological compartment of steroid. In regards to the former possibility, when appropriate corrections were made for artifactual dissociation of *in vitro* labelled receptors (Chapter III, Fig. 3.2) only a small percentage of the bound steroid dissociated as a result of passage through Sephadex G-25 columns. Assuming then that the latter possibility is valid, it is clear that either free steroid is derived from the steroid-protein complex or it enters the nucleus through other unidentified pathways.

Despite the lack of complete parallelism in the kinetics of incorporation of steroids by nuclei and of the accumulation of steroid-receptors by nuclei (Chapter V, Fig. 5.5), these results are compatible with the view that the development of the compartment of free steroid is conditional to the appearance of steroid receptors complex in the nucleus. Since the 8-fold increment in total nuclear steroid between 10 and 120 min (Fig. 5.5A) is nearly equal to the concomitant 10-fold increment in binding (Fig. 5.5B), and since the relative proportion of free steroid is usually maintained at about 70% of the total intranuclear steroid, it is therefore possible to define a relationship between free steroid and the amount of binding. Such a correlation is not surprising if the uptake of steroids by nuclei is dependent on an active transport process where there is a phase of rapid dissociation of the carrier-steroid complex. The dependence of intracellular transport on the carrier function of steroid-receptors is also suggested by several lines of evidence obtained from work on hormone sensitive and insensitive cells showing that the uptake of steroids by nuclei is not achieved in the absence of appropriate binding proteins (Bullock and Bardin, 1970; Baxter et al., 1970; Ohno et al., 1971; Bullock et al., 1971; Rosenau et al., 1972; McGuire et al., 1972; Shyamala, 1972; Goldstein and Wilson, 1972).

A peculiarity of the androgen carrier-protein is that it fails to form a complex with any potent natural androgen other than testosterone or dihydrotestosterone. Biologically active compounds such as androstanediol, androsterone, androstanedione and androstenedione are seldom found in the prostatic nucleus (Bruchovsky, 1971), as a rule do not bind to cytosol receptor, and probably undergo enzymatic conversion to dihydrotestosterone before they are active (Bruchovsky, 1971). One may reasonably suspect therefore that the intracellular transport of androgens is conditional on the nature of the substrate as well as on the availability of suitable carriers.

When the cytosol receptors, labelled under pulse-chase conditions, were chromatographed on cellulose phosphate columns the protein bound radioactivity was found exclusively in the Peak 2 fractions. However, when these extracts were chromatographed on Sephadex G-200 the resultant profile of radioactivity was different than that observed in pulse experiments (Fig. 6.2). In addition to Sephadex Peak I and Peak II complexes ^{3}H-steroids were also found in association with Sephadex Peak III receptors. Thus it appeared that the label was chased either from Peak III to Peak II and Peak I receptors, or conversely, from the Peak II and Peak I to Peak III receptors. Since in pulse experiments most of the radioactivity in nuclei is recovered in the Sephadex Peak III fraction (Fig. 5.7), and since there is considerable evidence to suggest that the nuclear receptor originates in the cytoplasm it is more likely that cytosol Peak III receptor is formed from cytosol Peak II (or Peak I) receptors.

Examination by column chromatography of the nuclear receptors formed in the pulse-chase experiments revealed that when the chase interval was extended to 60 min there was an increase in the relative proportion of ^{3}H-steroid recovered in Sephadex Peak I as compared to that found in Sephadex Peak III (Fig. 6.5). After a chase of 20 min most of the bound radioactivity was observed in Peak 2a fraction obtained by cellulose phosphate chromatography (Fig. 6.4). The results are therefore compatible with the idea that there is a steroid-acceptor or a steroid-receptor-acceptor site in the nucleus which is distinct from the steroid-receptor. The latter appears to function as a carrier molecule in the transport of androgens from cytoplasm to nuclei.

A final point that merits emphasis is the finding that estradiol is incorporated by prostatic nuclei and appears to be bound to the same receptor which binds testosterone and dihydrotestosterone (Table 6.1). Since estradiol is not known to mimic the action of androgens on prostatic epithelial cells, it seems that the presence of receptor in the nucleus in the absence of testosterone and dihydrotestosterone is not a sufficient stimulus to initiate a physiological response. Because the data suggest that the action of cyproterone acetate is similar to that of estradiol (Table 6.4) consideration should be given to the possibility that the action of anti-androgens such as estradiol and cyproterone acetate is based on their ability to induce the formation of inactive complexes which migrate into the nucleus. Saturation of the nucleus with such complexes or the resultant depletion of receptors in cytosol would account for certain aspects of chemical antagonism to androgens. Other compounds such as epitestosterone may produce similar effects.

CHAPTER VII

DISCUSSION

This investigation was designed to elucidate two aspects of the early action of androgens in rat prostate. One principal objective was concerned with the identification of intracellular-receptors and the homology between cytoplasmic and nuclear forms. A second principal objective was concerned with the function of steroid-receptors in cytoplasm and nuclei. The results of _in vitro_ experiments presented in Chapters III and IV provided evidence that steroid-receptors assume several configurations in tissue and that not all forms are common to both the cytoplasm and nucleus. Furthermore, the results of _in vivo_ experiments presented in Chapters V and VI indicated that there are fewer configurations _in vivo_ than _in vitro_. The use of pulse-chase experiments as described in Chapters V and VI established that certain of the steroid-receptors appear to function as carrier molecules in the transport of testosterone and dihydrotestosterone across the nuclear membrane. In the following discussion, these observations and related conclusions are reviewed in detail.

1. _In Vitro_ Studies

Results of _in vitro_ experiments indicated that at least 4 types of receptor proteins were present in prostatic cytosol. These were distinguished on the basis of their relative elution characteristics from cellulose phosphate and Sephadex G-200 columns. The chromatography of cytosol protein labelled *in vitro* with [1,2-^{3}H] dihydrotestosterone yielded two peaks of radioactivity (Peaks 1 and 2 in Fig. 4.1). When these peaks were reincubated and then subjected to Sephadex G-200 chromatography it was found that cellulose phosphate Peak 1 contained a receptor protein which had an apparent Stokes' radius of 48 Å (Fig. 4.7A). Cellulose phosphate Peak 2, on the other hand, contained three receptor complexes (Sephadex Peaks I, II, and III) which had Stokes' radii of 24 Å, 48 Å, and > 48 Å (Fig. 4.7B).

The chromatography of nuclear extract labelled *in vitro* with [1,2-^{3}H]dihydrotestosterone yielded two receptor complexes. One was eluted from cellulose phosphate as Peak 2a (Fig. 4.8) and from Sephadex G-200 as Peak III (Fig. 4.10A); the other was recovered as cellulose phosphate Peak 1 and Sephadex Peak I. The first had a Stokes' radius of approximately 24 Å, whereas the second had a Stokes' radius of > 48 Å. Thus under *in vitro* conditions six types of androgen-receptors were isolated from prostatic cells. None of these were found to correspond to steroid-binding proteins of the serum (Fig. 4.15 and 4.16).

2. *In Vivo* Studies

Results obtained from *in vivo* experiments differed from those obtained *in vitro* in that fewer receptor complexes were isolated under *in vivo* conditions. After a pulse injection of [1,2-^{3}H]-

testosterone into castrated rats only cellulose phosphate Peak 2 (Fig. 5.2) and the corresponding Sephadex Peaks I and II (Fig. 5.3) were isolated from the cytosol (note the absence of Sephadex Peak III). The relative amount of radioactivity associated with Sephadex Peaks I and II was determined by the ionic strength of the eluant. This effect of ionic strength was also seen _in vitro_ (Fig. 4.2) and in general, is comparable to the transition of the 8S receptor to the 3S form reported by others (Baulieu and Jung, 1970; Jung and Baulieu, 1971). Although cellulose phosphate Peak 1 receptor was not seen _in vivo_, trace amounts of Sephadex Peak III complex were observed during pulse-chase experiments _in vivo_ (Fig. 6.2).

Nuclear extracts examined less than 60 min after the _in vivo_ administration of [1,2-^{3}H]testosterone mainly yielded Sephadex Peak III receptor corresponding to a Stokes' radius of 24 Å (Fig. 5.6). At later intervals, however, a significant amount of radioactivity was also bound to the larger form of nuclear receptor (Fig. 5.7). It is noteworthy that the nuclear receptors demonstrated under _in vivo_ conditions using castrated rats are similar to those demonstrated under _in vitro_ conditions using non-castrated rats. However, as will be pointed out later, different results are obtained _in vitro_ when nuclei from castrated rats are used.

3. The Incorporation of Androgens into Nuclei and Active Transport

In classical terms active transport involves the following steps (Kaback, 1972):

(1) Substrate combines with a specific carrier on one side of a membrane and is translocated to the other side of the membrane.

(2) The complex undergoes a conformational change so that the substrate has a reduced affinity for the carrier and is released.

(3) The net result of this mechanism is that substrate is concentrated against a chemical gradient at the expense of metabolic energy.

The transport of testosterone and dihydrotestosterone into prostatic nuclei appears to depend on a system which possesses these characteristics. First, testosterone and dihydrotestosterone accumulate in nuclei against a chemical gradient (Figs. 5.1 and 5.5). Secondly, the rate at which these steroids are transported seems to be limited (Table 6.1). Thirdly, it appears that the steroids are transported into the nucleus by a process which depends on the carrier function of steroid-receptors (Figs. 6.1B and 6.3B). Finally it appears that the steroid-receptor is modified slightly during passage across the nuclear membrane (Figs. 5.2 and 5.6). This alteration presumeably results in a change of affinity and partial dissociation of the complex. The observation that a significant amount of intranuclear steroid is in the free form is in keeping with the latter conclusion.

Although in this investigation it was not shown that nuclear uptake of hormones is energy dependent, Sar et al. (1970) reported that prostatic minces concentrate steroids in the nucleus when the incubation temperature is 37° C but not when the temperature is 2° C. Rochefort and Baulieu (1969) demonstrated that the uptake of estradiol into uterine nuclei is also a temperature dependent process.

While nuclear uptake and retention of androgens operates through a process which is characteristic of active transport, the passage of steroids through the plasma membrane of prostatic cells occurs by way of a different mechanism. In this investigation it was found that the amount of steroid entering the cytosol fraction was directly proportional to the dose of injected steroid (Table 6.1). Since even at relatively high doses of injected steroid (250 μg) it was not possible to saturate the transport capacity of the plasma membrane, it seems reasonable to believe that this process is not carrier mediated. Thus in accordance with the conclusions of other investigators (Giorgi et al., 1971; Munck, 1971; Brinkmann et al., 1972) it appears that steroids enter prostatic cells by passive diffusion.

4. The Carrier Function of Steroid-Receptors

Two observations indicate that cytosol receptors of the rat prostate are indeed essential for the incorporation of androgens into nuclei. First, it was demonstrated that the amount of ^{3}H-steroid chased from cytosol receptors to the nuclear receptors was quantitative and that the transfer occurred sequentially (Figs. 6.1B and 6.3B). Secondly, despite the presence of at least five androgen metabolites in the cytoplasm (Bruchovsky, 1971), only testosterone and dihydrotestosterone were found to bind to cytoplasmic receptors (Fig. 5.1B); this limitation in binding corresponded to the limitation on the types of androgens transported into the nucleus. These findings are consistent with the view that either the steroid moiety of the cytosol steroid-receptor complex is transferred to nuclear receptor, or alternatively, that the entire cytosol complex enters the prostatic nucleus. As discussed below the evidence obtained in this study tends to favour the latter alternative.

When binding studies were performed *in vitro* with nuclear extracts obtained from castrated animals (Fig. 4.9 and 4.10B) only the larger form of receptor was evident (Sephadex Peak I). However, when castrated rats were injected with $[1,2-^{3}H]$testosterone (Fig. 5.6 and 5.7) there was a progressive increase in the level of Sephadex Peak III receptor complex (24 Å form). Since this complex is only seen in the nuclei of androgen-stimulated prostates (Fig. 4.8 and 4.10A) it seems reasonable to believe that this receptor complex is transferred from the cytoplasm. The fact that certain of the cytoplasmic receptors have chromatographic properties resembling those ascribed to the small nuclear receptors lends further support to this view. It is clear that the small 24 Å forms of nuclear and the cytoplasmic complexes both display similar affinity for cellulose phosphate (Fig. 5.2 and 5.6). Moreover, the Sephadex Peak III receptors which are generated in cytosol under *in vitro* conditions, would appear to be the same as the Sephadex Peak III receptors which appear in nuclei under *in vivo* conditions. These results are consistent with reports that both nuclear and cytoplasmic receptors have sedimentation coefficients of about 3-4S (Fang *et al.*, 1969; Fang and Liao, 1971; Mainwaring and Peterken, 1971).

A final point which tends to support carrier role assigned to cytosol receptors is derived from studies with reconstituted cell free systems. In such investigations it has been shown that 3-4S steroid-receptor complexes formed in the cytosol *in vitro* are able to permeate the nuclear membrane when incubated with isolated nuclei (Fang *et al.*, 1969; Fang and Liao, 1971).

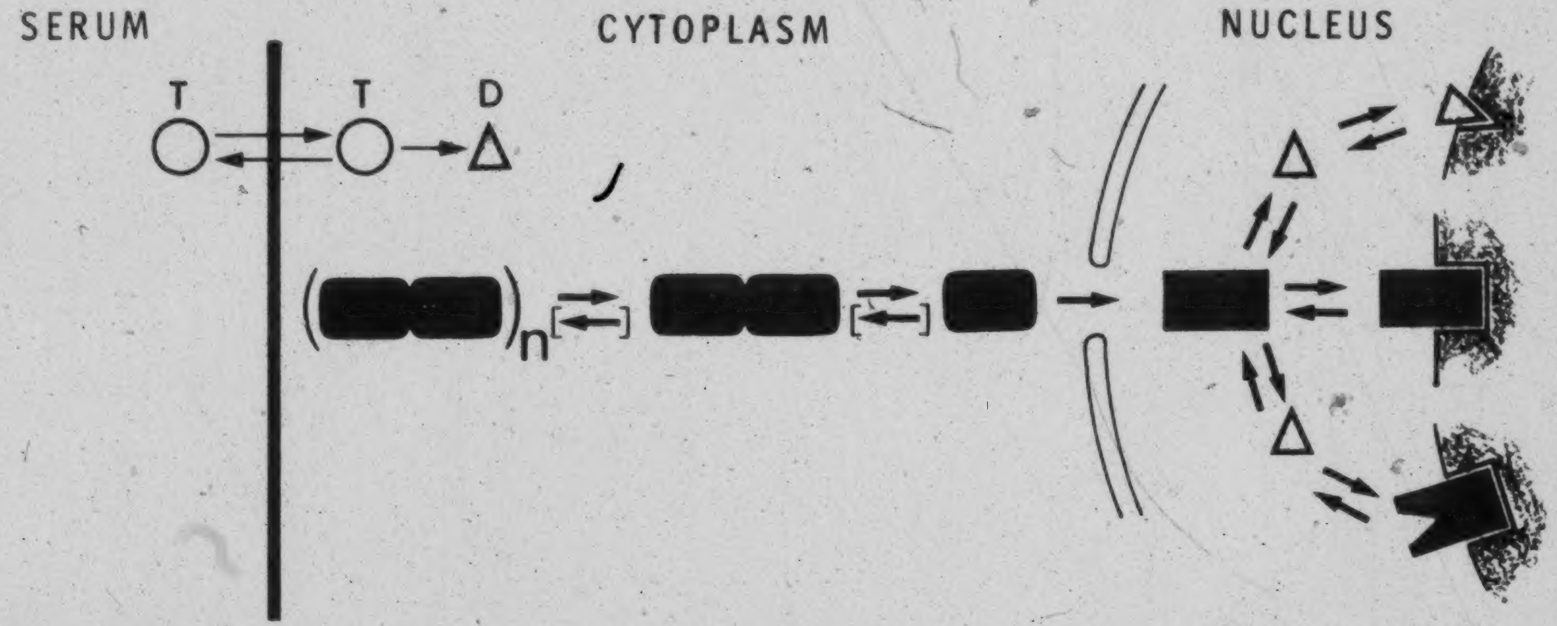
5. The Presence of Acceptor Sites in Prostatic Nuclei

At present it is generally believed that after the receptor complexes pass into the nuclei of target cell tissues they bind to acceptor sites within the chromatin (King and Gordon, 1972); Musliner and Chader, 1971; Tymoczko and Liao, 1971; Liao *et al.*, 1973). While evidence in support of this hypothesis has been difficult to obtain, three findings made in this investigation are compatible with existence of acceptor molecules in prostatic nuclei. First, results from *in vitro* studies suggested that prostatic nuclei possess a class of androgen binding sites (i.e. Sephadex Peak I) that are not grossly affected by castration (Fig. 3.6 and 4.10). Secondly, under pulse and pulse-chase conditions the levels of radioactivity associated with these sites increased as a function of time after the injection of [1,2-^{3}H]testosterone (Fig. 5.7 and 6.5). Thirdly, when *in vivo* labelled nuclei were frozen prior to examination on Sephadex G-200 only the Sephadex Peak I sites were labelled with radioactive hormone (Fig. 5.8). Whether the observed binding of ^{3}H-androgens to Sephadex Peak I was due to the binding of free steroids or whether it arose as a result of binding of smaller receptor complexes (i.e. Sephadex Peak III) is not certain. Also the possibility that Sephadex Peak I radioactivity represents a non-specific aggregate of binding proteins cannot be completely discounted.

6. A Scheme of the Function of Receptor Proteins in the Intracellular Transport of Androgens

On the basis of the apparent structural similarities and functional relationships ascribed to the receptor proteins it is possible to construct a model which outlines the role of steroid-receptors in the transport of steroids from the cytoplasm to the nucleus (Fig. 7.1). Testosterone, the principal male sex hormone diffuses from the circulation into the prostatic cell and is metabolized to dihydrotestosterone and other unconjugated androgens. Dihydrotestosterone and unmetabolized testosterone then bind to Sephadex Peak I receptor, which is probably equivalent to the 8S receptor described by Mainwaring and Peterken (1971). After this event the newly formed

A MODEL FOR RECEPTOR PARTICIPATION IN THE NUCLEAR UPTAKE OF ANDROGENS



IN VIVO BINDING	T;D	T;D	-	T;D	(T;D)
IN VITRO BINDING	T;D	D	T;D	T;D	T;D
SEDIMENTATION COEFFICIENT	8S	3-4S	3-4S	3S	-
STOKES' RADIUS (A)	-	48	24	24	-
CELLULOSE PHOSPHATE PEAK	2	2	2	2a	1
SEPHADEX G-200 PEAK	I	II	III	III	I

receptor complex undergoes a transition step to yield Sephadex Peak II complex. It is conjectured that Sephadex Peaks I and II may be related through an equilibrium reaction.

Next a second transition yields a smaller receptor (Sephadex Peak III) which penetrates the nuclear membrane. Although the factors regulating the formation of this complex are unknown, there is some evidence which suggests that a specific enzyme may catalyze this type of reaction (Puca *et al.*, 1972). Because the concentration of Sephadex Peak III complex is very low *in vivo* it seems reasonable to suspect that this species has only a transitory existence in the cytoplasm.

During, or shortly after its passage through the nuclear membrane, Sephadex Peak III complex is modified so that it now has a reduced affinity for its substrate but binds more tenaciously to cellulose phosphate. Androgens appear in a bound and free state in the nucleus and it is not clear which form, if either, is active.. It can be visualized that free steroid interacts directly with chromatin, that steroid-receptor complex interacts with chromatin or that the receptor (minus steroid) is the activator molecule. A final question yet to be elucidated concerns the fate of the intranuclear steroid; further study of the mechanisms involved in the discharge of steroids from the nucleus might provide useful information on the significance of various associations of steroids and receptors in prostatic cells.

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APPENDIX

A. Estimation of Protein Concentration from Absorbance at 280 nm and 260 nm (Layne, 1957).

Protein concentration (mg/ml) = $1.55\ D_{280} - 0.76\ D_{260}$

where: D_{280} is the absorbance at 280 nm

D_{260} is the absorbance at 260 nm.

B. Estimation of Standard Error of the Mean (S.E.)

$$s = \sqrt{\frac{\sum_{1}^{n} (Xi-\overline{\chi})^2}{n-1}}$$

$$S.E. = \frac{s}{\sqrt{n}}$$

where: s is the sample variance

n is the sample size

$\overline{\chi}$ is the sample mean

S.E. is the standard error.

C. Calculation of the Association Constant (Ka) (Scatchard, 1949).

$$\frac{\overline{v}}{(A)} = Ka(n-\overline{v})$$

where: $\bar{v}$ is the amount bound (moles/mg)

(A) is the amount unbound (moles/ℓ)

Ka is the association constant

n is the number of sites (per mg)

D. Publications Arising from this Research:

Abstracts

1. RENNIE, P. & BRUCHOVSKY, N., Comparison of the intracellular binding of testosterone and dihydrotestosterone. Proceedings of the Canadian Federation of Biological Societies 14, 237 (1971).

2. RENNIE, P. & BRUCHOVSKY, N., Effect of binding proteins on androgen localization in target tissues. IV. International Congress of Endocrinology, Abstracts of Short Communications pp. 79 (1972).

3. RENNIE, P. & BRUCHOVSKY, N., Turnover of protein bound androgens. Proceedings of the Canadian Federation of Biological Societies 15, 412 (1972).

4. RENNIE, P. & BRUCHOVSKY, N., Androgen uptake by prostatic nuclei. Clin. Research XX, 923 (1972).

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1. RENNIE, P. & BRUCHOVSKY, N., *In vitro* and *in vivo* studies on the functional significance of androgen receptors in rat prostate. J. Biol. Chem. 247, 1546 (1972).

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3. BRUCHOVSKY, N., LESSER, B. & RENNIE, P., Control of the concentration and distribution of dihydrotestosterone in prostatic cells. In "Normal and Abnormal Growth of the Prostate", to be published.